BIOLOGICAL CONTROL OF POSTHARVEST DISEASE IN THE PERISHABLE FRUIT INDUSTRY BY *BACILLUS* LIPOPEPTIDES

By

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DECLARATION

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ABSTRACT

The work presented in this study aimed to investigate the suitability of *Bacillus* spp. lipopeptides to be used as effective biocontrol agents that have the potential to replace chemically synthesised pesticides that are currently in use in post-harvest disease control. The screening criteria were based on the ability of different *Bacillus* species to produce antifungal lipopeptides which in turn could inhibit phytopathogens isolated from post-harvest grapes.

The study further aimed to identify the lipopeptides responsible for any noted antifungal activity and investigate possible downstream isolation methods that could be included into process optimisation. With these goals in mind, the work presented here hopes to inform on the way forward for future studies that are focused towards the controlled in vitro production of a customised cell-free biocontrol agent for post-harvest disease control.

A phytopathogen culture bank was established from 79 crude isolates of fungi from post-harvest grapes supplied by the South African Table Grape industry. These isolates were purified into 59 pure cultures using 5 sequential rounds of sub-culturing on PDA, MEA and NA agar plates. Of these, 16 isolates were successfully sequenced using Sanger sequencing with ITS 4 and ITS 5 primers.

Of the identified phytopathogens, 50% belonged to *Penicillium* spp., 33% to *Botrytis* spp. and the remaining 17% grouped as *Aspergillus* spp., *Alternaria* spp. and *Lewia* spp. These phytopathogens are responsible for noble rot, blue-green mould, bunch rot, black mould and leaf blight in the grape industry respectively.

Four different strains of lipopeptide producing bacteria, *Bacillus amyloliquefaciens* DSM 23117, *B. licheniformis* DSM 13, *B. subtilis* ATCC 21332 and *B. subtilis spizizini* DSM 347 were obtained from culture collections and screened in triplicate in shake flasks over a seven day period using media from Kim et al (1997) with modifications. *Bacillus amyloliquefaciens* DSM 23117 was identified as the superior producer of lipopeptides.

In addition to the significant amounts of lipopeptides produced by this strain, crude lipopeptides from *B. amyloliquefaciens* were shown to exhibit efficacy (either by causing death or static inhibition) towards all 9 isolated fungal phytopathogen strains tested in radial diffusion assays (RDA). The strains incorporated in RDA assays belonged to *Botrytis*, *Penicillium*, *Alternaria*, *Aspergillus* and *Lewia* genera.

TLC separation in combination with antifungal efficacy assays further narrowed down the Iturin family, peaks 3, 4, 5, 8 and 9, as compounds that exerted significant antifungal activity. Thus indicating these compounds are important targets for isolation and incorporation into antifungal studies towards the development of cell-free biocontrol products for use in post-harvest applications and crop protection.

Using different downstream methods for lipopeptide isolation and purification, salt precipitation was identified as a bulk isolation method while solvent extraction from isobutanol and n-hexane proved superior in separating antifungal lipopeptides from surfactin.

Through the use of various analytical and experimental methods all the aims set out in this study were met by large. *B. amyloliquefaciens* was identified as a target strain for culture and process optimisation, targeting iturins, and for incorporation towards the development of a cell-free biocontrol agent targeting phytopathogens found in, but not limited to, the South African Table Grape Industry.

UITREKSEL

In die studie is die bakterium *Bacillus* spp bestudeer as moontlike produseerder van anti-fungiese middels wat as plaasvervanger kan dien vir die huidige chemiese middels wat as plaagdoders gebruik word teen fungi. Die kriteria waaraan die siftingstoets onderworpe was, is gebasseer op die vermoeë van die bakterium om anti-fungiese middels te kon produseer en/ of hierdie middels anti-fungiese aktiwiteit kon toon teen die fungi wat ge-isoleer is uit die natuur, meer spesifiek, tafeldruiwe.

Met die studie is verder gepoog om die anti-fungiese middels, lipopeptiedes, wat verantwoordelik is vir anti-fungiese aktiwiteite, te identifiseer asook moontlike prosesse te identifiseer vir isolasie. Met die doelwitte word beoog om toekomstige navorsing te lei wat fokus op die in vitro produksie van ‘n selvrye produk wat kan dien as ‘n biologiese plaagdoder.

‘n Kultuurbank vir anti- fungiese eksperimente is geskep vanuit 79 fungiese monsters wat vanaf die Suid-Afrikaanse Tafeldruifbedryf verkry is. Dit is verder omskep in 59 suiwerkulture. DNS identifikasie is gedoen op 18 kulture en 16 is suksesvol geidentifiseer met behulp van DNS herhalings en morfologie studies. Die fungi is gegroep as 50% *Penicillium* spp., 33% to *Botrytis* spp. en 17% *Aspergillus* spp., *Alternaria* spp. en *Lewia* spp. saamgegroepeer.

Vier spesies van *Bacillus* naamlik *Bacillus amyloliquefaciens* DSM 23117, *B. licheniformis* DSM 13, *B. subtilis* ATCC 21332 en *B. subtilis spizizini* DSM 347 is bekom vir die anti-fungiese middels wat hulle produseer, naamlik lipopeptiedes. Bacilluskulture is gekultiveer op selmedia soos gebubliseer deur Kim et al. (1997)

met modifikasies en *Bacillus amyloliquefaciens* is geidentifiseer as die top spesie vir lipopeptiedproduksie.

Die resultate van *B. amyloliquefaciens* gedurende kultivering dui daarop dat die spesie van *Bacillus* ‘n oorproduseerder is van anti-fungiese middels. Die ongesuiwerde anti-fungiese middels het ook sterk anti-fungiese eienskappe teen al 9 van die fungi-isolate waarteen dit getoets is, getoon. Die fungiese kulture getoets, het behoort aan *Botrytis*, *Penicillium*, *Alternaria*, *Aspergillus* en *Lewia* genera.

Dunlaag chromatografie in kombinasie met anti-fungiese toetse het Iturins, pieke 3, 4, 5, 8 en 9 geidentifiseer as die middels verantwoordelik vir anti-fungiese aktiwiteit wat die groep ‘n belangrike onderwerp vir verdere studie maak met die doel op die ontwikkeling van ‘n selvrye produk.

Deur gebruik te maak van verskillende isolasie- en suiweringsmetodes is soutpresipitasie geidentifiseer as ‘n metode vir algemene isolasie van alle lipopeptiedes terwyl isobutanol en n-hexaan spesifiek anti-fungiese middels geteiken het vir isolasie.

Deur die gebruik van verskeie analitiese- en eksperimentele metodes is al die doelwitte wat in hierdie studie uiteengesit is, behaal. *B. amyloliquefaciens* is geïdentifiseer as 'n teikenspesie vir verdere prosesoptimalisering Iturins en vir inkorporasie in studies vir die ontwikkeling van 'n selvrye, biologiese plaagdoder wat op fytopatogene in die Suid-Afrikaanse Tafeldruifbedryf fokus.

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DEDICATIONS

To Mom for all her personal sacrifices towards my education.

Thank you.

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ABBREVIASIONS

µM	*Micromolar*
ATCC	*American Type Culture Collection*
AU	*Arbitrary Units*
BLAST	*Basic Local Alignment Search Tool*
BSA	*Bovine Serum Albumin*
CDW	*Cell Dry Weight*
dH_2O	*Distilled Water*
DSM	*German Type Culture Collection*
EtOH	*Ethanol*
GRAS	*"Generally Regarded As Safe"*
HCl	*Hydrochloric Acid*
HPLC	*High Pressure Liquid Chromatography*
IC	*Ion Chromatography*
ITS	*Internal Transcribed Spacers*
M	*Molar*
MEA	*Malt Extract Agar*
MIC	*Minimum Inhibition Concentration*
mM	*Millimolar*
NA	*Nutrient Agar*
NB	*Nutrient Broth*
nm	*Nanometer*
OD	*Optical Density*
OD_{620}	*Optical Density at 620nm*

PCR	*Polymer Chain Reaction*
PDA	*Potato Dextrose Agar*
PDB	*Potato Dextrose Broth*
RP	*Reverse Phase*
RPM	*Revolutions Per Minute*
SATGI	*South African Table Grape Industry*
SO_2	*Sulphur Dioxide*
Spp	*Spesies*
TBME	*tert-Butyl-methyl-ether*
TLC	*Thin Layer Chromatography*
TSB	*Tryptic Soya Broth*
UV	*Ultraviolet*

Chapter 1

GENERAL INTRODUCTION

Establishing and ensuring food-security has become vital as the world faces overpopulation and the depletion of our natural resources. Recent studies (Future Directions International (Pty) Ltd., 2011) estimates that almost a third of the agricultural crops produced worldwide for human consumption, around 1.3 billion tons, are lost every year. Of these losses, as much as 40% result from pests such as phytopathogens (an organism that is pathogenic to plants), weeds and/or invertebrates (Glare, et al., 2012).

Not only are these losses costly due to the loss of the product and production cost thereof, but pathogens affecting fruit also present a significant risk to human health. This is especially true in poorer countries where contaminated food is consumed.

Reduction of crop losses has been largely dependent on chemically derived antagonists. Unfortunately, these have been shown to have undesirable effects on the environment as well as on human health due to their toxicity (Pimentel, 2005). Increased resistance to chemical pesticides from the over use of chemicals has also increased dramatically over the past few years and poses a severe risk to farmers and their crops.

Biological antagonists, one example being lipopeptides, on the other hand have been proposed as an innovative, effective and ecologically benign control strategy for the reduction of crop losses, particularly those resulting from postharvest decay.

Despite its seeming superior effectiveness compared to chemical control at lower effective doses and low toxicity and low bioaccumulation however, to date only 2.5% of the global pesticide market comprises of biological control agents. This is attributed to the high production costs, scant knowledge of their synthesis and lack of optimisation routes for production. Much research is still needed. This makes biological pesticides and attractive subject for research to advance our understanding thereof and develop an economical green alternative to chemical pesticides in the form of cell-free biocontrol agents.

1.1 PROJECT OUTLINE

The scope of the study presented in this thesis was directed towards evaluating the effectiveness of naturally occurring, biologically synthesised *Bacillus* spp. lipopeptides as alternative novel biocontrol agents, as opposed to chemically synthesised pesticides currently in use, to act against phytopathogens responsible for postharvest disease found in, but not limited to, the South African table grapes industry.

Furthermore, the study aims to also inform on lipopeptide production, the specific lipopeptide homologs responsible for the observed antimicrobial activity against phytopathogens and on possible methods for lipopeptide isolation and purification. Answers to these questions would ultimately elucidate the direction to be taken for future investigations that are focused towards process optimisation and scaling up strategies of *Bacillus* lipopeptides as biocontrol agents.

To achieve the goals for the study set out above, the following four aims and objectives were set as the criteria to be met in order to validate the ability of *Bacillus* lipopeptides as an effective antimicrobial compounds.

- Aim 1: Establish a phytopathogen culture bank from South African table grapes for efficacy studies.
    - Isolate phytopathogens from infected grapes obtained from SATGI (South African Table Grape Industry).
    - Purify crude isolated cultures until pure phytopathogen cultures are obtained.
    - Identify obtained phytopathogens cultures.
    - Maintain a culture bank throughout the duration of experiments.

- Aim 2: Establish a *Bacillus* spp. culture bank for lipopeptide production.
    - Identify candidate *Bacillus* strains through a literature study with the focus on documented expression of specific lipopeptide homologs by each strain.
    - Obtain the identified *Bacillus* strains through DSZM and ATCC.
    - Maintain a culture bank of *Bacillus* strains throughout the duration of experiments.

- Aim 3: Determine the efficacy of *Bacillus* lipopeptides produced by each *Bacillus* strain towards phytopathogens isolated from South African table grapes.
    - Identify a suitable growth media through literature study.
    - Culture each of the candidate *Bacillus* spp. via the use of shake flasks.
    - Isolate crude media samples from shake flasks during bacterial growth of each *Bacillus* strain.
    - Screen lipopeptide homologs and measure the quantity of each lipopetide produced during bacterial growth.
    - Measure and quantify the efficacy of produced *Bacillus* lipopeptides towards phytopathogens corresponding to each sample from shake flasks during culturing.
    - Identify a test strain for bioautographic assays.
    - Perform MIC study to determine the minimum inhibition concentration of crude lipopeptides.

- Aim 4: Identify which lipopeptide homologs are responsible for any activity observed towards the phytopathogen strains tested using bioautographic assays.
    - Mathematically correlate lipopeptide production and efficacy towards phytopathogens.
    - Isolate lipopeptides from crude media samples through acid precipitation, salt precipitation and organic solvent extraction.
    - Plate lipopetide extracts on TLC plates.
    - Observe spots on one plate using UV and chemical detection techniques.
    - Overlay TLC plate with PDA inoculated with a phytopathogen test strain found vulnerable towards lipopeptides in RDA studies.
    - Measure R_f of active sites on TLC plates.
    - Extract lipopeptides from each R_f band and identify lipopeptide homologs present.

Chapter 2

LITERATURE REVIEW

There is a growing demand for green and environmentally friendly alternatives for post-harvest disease control. It is estimated that around the world about 1.3 billion tons of food is wasted annually either through post-harvest disease, improper storage conditions and/or handling (Thomas, 2011). Of these losses as much as 40% have been linked to post-harvest phytopathogens, i.e. pathogens to plants, as the causative agent (Oerke & Dehne, 2004; Prusky, 2011; Glare, et al., 2012). To put this figure into perspective, this loss comprises enough food to feed our current global population and for this reason causes major concern if we are to feed the estimated 10 billion people expected to populate the earth by 2050 (Kumar, 2015). Post-harvest losses are also expensive since they not only include the wasted product, but also in turn the loss in production, cost of labour, fertilization, irrigation and spent resources during cultivation.

The term “Biopesticide” is used to describe any biological product, be it the live organism or their metabolites, that has the ability to exert antimicrobial activity against pests that could be from bacterial, viral, fungal, insect, protozoan, plant etc. sources. Using the humble ladybug as an example, the lady bug is the natural enemy of aphids and larvae responsible for crop losses and low crop yields. In this context, applying ladybugs bred in captivation to crops during cultivation makes

ladybugs the “biopesticide” towards the control of insect pests and minimising plant damage.

Biopestecides have been proposed as biocontrol agents to replace chemical control of crop pests that result in food losses during cultivation and post-harvest. This thesis will focus on the post-harvest protection of grapes through biologically produced *Bacillus* metabolites, called cyclic lipopeptides, which have shown promise as effective biopesticides in the emerging biopesticide market through research findings.

In this chapter synthetic pesticides will be discussed with the focus on the drawbacks that are resulting from using these chemicals and how this underscores the need for alternative methods in post-harvest disease control. Crop losses in grapes resulting from different fungi will be highlighted for identifying the known targets for new biocontrol agents. *Bacillus* lipopeptides will be discussed next and how lipopeptides demonstrate the potential, specifically in table grapes, to be used as alternative biocontrol agents in post-harvest applications.

To further evaluate *Bacillus* lipopeptides as biocontrol agents it is also important to compare large scale production strategies for lipopeptides and the cost involved. Production strategies to ensure low production cost is needed if lipopeptides are to compete with the relatively low cost of synthetic pesticides through chemical synthesis, which are established products in the pest control market. As such, current strategies employed in research towards establishing a cost-effective cultivation strategy and low cost product will be investigated and how these are being employed on a lab scale in an attempt to develop low cost industrial fermentation processes to compete with chemical synthesis.

2.1 SYNTHETIC PESTICIDES DOMINATE THE MARKET

For any farmer it is important to protect crops during cultivation to ensure the highest yield and financial return. Reduction of crop losses has been largely dependent on chemically derived antagonists that combat pests and diseases during cultivation and postharvest storage.

Crop protection through the use of insecticides can be traced back as far as 1500 BC when Egyptians used them to repel aphids, fleas and other insects (Kesavachandran, 2014). During the 1600's mixtures of honey and arsenic was applied to kill ants and by the end of the 19th century farmers where using a variety of arsenic compounds to protect their crops (Thakore, 2006). The breakthrough for synthetic pesticides came in 1939 when Paul Muller discovered the pest control abilities of the organochlorine Dichlorodiphenyltrichloroethane (DDT) (Thakore, 2006), today a known mutagen for causing genetic defects in mammals (Qiu, 2013). Since its synthesis and undiscriminating application DDT has led to the contamination of numerous water and food supplies from overuse and was subsequently banned from use (Kumar, 2015). Another major advancement for chemical pest control was parathion, a potent organophosphate insecticide discovered by German scientists experimenting with nerve gas and still applied widely today (Thakore, 2006). DDT and organophosphates together with Benzene hexachloride (BHC), aldrin, dieldrin and endrin was used throughout the 1950's to 1970's because of their broad-spectrum biocidal activity against a variety of phytopathogens encountered by farmers (Thakore, 2006).

Recent advances seen in food production over the last 50 years have been mainly attributed to the usage of vast quantities of chemically derived synthetic pesticides, which, arguably, is no longer sustainable (Oerke, 2006; Glare, et al., 2012). Globally $26.7 billion is spent developing synthetic chemical agents for crop management (Figure 2-1) and toxicology studies to ensure human safety adds to this bill at the end of the day (Thakore, 2006) In Figure 2-1 the move to green alternatives can be seen increasing as pressure from government and end-users increase towards organic production and green farming techniques.

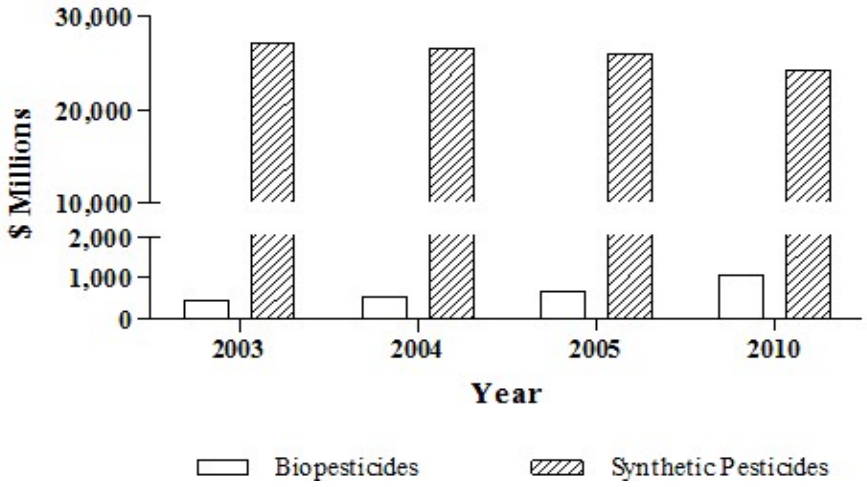

Figure 2-1 Global pesticide market statistics by year comparing the growth of biopesticides and current synthetic pesticide usage. (adapted from Thakore, 2006)

Moreover, however great the achievement for chemical pest control may be, there is nevertheless sufficient evidence of the adverse effects synthetic pesticides exert on the environment and human health (Pimentel, 2005; Thakore, 2006). These chemicals are hazardous to humans and other species, either directly through exposure during application, contamination of items or indirectly through harmful residues left behind in treated soil, food and/or underground water supplies (Kumar, et al., 2008) leading to bioaccumulation. Up to 40% of food tested by the World Health Organisation contained harmful pesticide residues (Maksimov, et al., 2011).

Not only do synthetic compounds kill off the unwanted pathogens they are meant to eliminate, but due to their inherent broad biological toxicity the chemicals and their residues left behind after application indiscriminately eradicate other valuable organisms already present in the niche (Maksimov, et al., 2011). Examples of these include beneficial birds that eat locusts feeding on crops, insects such as the humble ladybug living on a diet of aphids and the loss of irreplaceable bees that pollinate fruit trees. Also implicated are root and soil colonising micro-organisms. All these species which are beneficial to the ecosystem are adversely affected by chemical pesticide application.

Synthetic pesticides stimulate resistance development in the phytopathogens being treated and subsequently require either higher doses of the pesticide to achieve the

same level of activity and/or new alternative formulations to be developed (McGrath, 2001; Fernández-Ortuño, et al., 2006; Banat, et al., 2010). The approach by humans to apply higher doses or new formulations to combat resistant pests is a double edged sword since applying higher doses to counteract the resistance developed results in an increased resistance towards the old or new chemicals (Van den Bosch, et al., 2011).

The adverse effects caused by synthetic pesticides from indiscriminate crop application over the last 50 years has seen the introduction of strict regulation and laws, nationally and internationally, regarding production, trade and application of these compounds for crop management (Holzmann, 2010; Thakore, 2006) . Thus, due to the negative connection to chemical pesticides and pressure from governments, consumers are also becoming more environmentally aware and are pursuing organic products which put pressure on farmers to find green alternatives to conventional crop management strategies.

In summary, synthetic pesticides has remained the default strategy for pest management and still remains the choice towards pest control due to their large presence in the market and time-proven effectiveness. Chemically derived pesticides have however had significant and unfavourable consequences, causing damage to the environment and have seen the rise of chemical resistance developing in pests from continuous application.

2.2 POST-HARVEST PATHOGENS AFFECTING GRAPES

As one of the most significant and economically exported/imported fruit in the world, grapes contribute an important part to the global fruit and wine market (Jiang, et al., 2014). However, even at 0°C during cold storage grapes are plagued by a variety of pathogens, mainly being fungal (Lichter, et al., 2002), such as *Penicillium* spp., *Aspergillus* spp., *Alternaria* spp., *Botrytis* spp. and *Rhizopus* spp. to name but a few. These post-harvest pathogens cause an estimated loss of about 39% in yield and 30% in value (Jiang, et al., 2014).

Currently the post-harvest losses are addressed by Sulphur Dioxide (SO_2) fumigation of cold storage rooms and grapes for export being stored with SO_2 lined generator sheets between crates (Lichter, et al., 2002). Both these fumigation and

generator sheet strategies are limited to cold storage and are dependent on a range of factors. The two main factors for effectiveness include achieving proper dispersion of the SO_2 gas between crates as well as sustaining sufficiently high SO_2 concentrations for successful inhibition of post-harvest disease during storage (Lichter, et al., 2002). Methods like these that contain high SO_2 levels are known to damage the grape berries leading to a lower grade product, cause an unpleasant aftertaste and are harmful to humans, especially those who display a low tolerance for SO_2 exposure. Additionally, treatment with SO_2 has not proven to be effective in all circumstances to prevent fungal decay from happening during storage. Such is the case with *Rhizopus stolonifer*, a black mould, that requires very high concentrations of SO_2 to achieve grape protection (Lichter, et al., 2002).

Not only does the SO_2 harm the fruit and lack the efficiency in some cases to prevent phytopatogen growth, but fungal decay caused by *Aspergillus* spp. affecting grapes post-harvest is also hazardous to human health due to the production of mycotoxins such as aflatoxins (Tsitsigiannis, et al., 2012) and ochratoxin A; a group 2B carcinogen believed to be responsible for causing cancer based on evidence from renal toxicology, mutagenicity and immunotoxicity studies (Pfohl-Leszkowicz & Manderville, 2007). These carcinogens contaminate all afflicted fruit and wine consumed by humans and poses a big threat to the global market.

It is thus needed to search for alternative replacement strategies that offer post-harvest protection of grapes against fungal growth and thus focus on biocontrol strategies offering antifungal activity is essential. Such control strategies should offer complete protection against various fungal phytopathogens, not degrade the product and should be effective regardless of the environmental factors such temperature. These factors will be discussed when reviewing current *Bacillus* products that make use of live cultures and how their effectiveness is influenced, among other, by temperature.

2.3 BIOPESTICIDES: THE VERSATILE MULTI-TOOL

An important factor for producers is to obtain a high yield from crops and this only comes from protection against pests as mentioned. The adverse effects on the

environment caused by synthetic pesticides, increased phytopathogen resistance development, implementation of strict regulations and consumers seeking "organic" products has pressed scientists towards directing their research efforts on augmenting organisms and their molecular constituents for biocontrol strategies and improved biocontrol formulations.

With the damage current chemicals have caused in mind, the design for the ideal next generation pesticides should aim to kill off the targeted phytopathogens with minimal effect on the environment and other beneficial organisms, such as birds, mammals, bees etc. coupled with low to no bioaccumulation being present.

Biopesticides are any living organism or the naturally occurring products derived from these organisms such as plants, bacteria, viruses or fungi which are exploited for their ability to suppress phytopathogens. The suppression is classified as non-toxic mechanisms that are considered to be an eco-friendly alternative to chemical application (Thakore, 2006; Glare, et al., 2012; Kumar, 2015). Due to their low toxicity and high environmental compatibitity, due in large to being biologically synthesised products or natural enemies of the pests they target, biopesticides meet the criteria for next generation pesticides.

Current methods being developed are biocontrol agents which are directly applied as liquid formulations (Janisiewicz & Korsten, 2002), which will be further discussed in section 2.4, containing live cultures. Liquid formulations hold the biggest share of approximately 60% in the biopesticide market (Thakore, 2006)(Table 2-1).

Table 2-1 Biopesticide market in $US millions compared by different formulations available (Adapted from Thakore, 2006).

Biopesticide Formulation	**2003**	**2004**	**2005**
Liquid based	280.8	337.2	403
Granuels	70.2	84.3	100.8
Powder	70.2	84.3	100.8
Pellets	46.8	56.2	62.2

Unfortunately, compared to chemical agents, only 2.5% of the pesticide market (Figure 2-1) represents biocontrol agents (Ongena & Jacques, 2008) and globally a mere 22 million hectares are organically cultivated (Thakore, 2006). With approximately 175 active registered biopesticide compounds in comparison to a staggering 27 144 synthetic pesticides (Thakore, 2006) and organic cultivation representing less than 1% of the world's crops (Kumar, 2015), there is tremendous growth potential for biocontrol agents to be researched, developed and incorporated in agriculture.

Keeping this open area for development in mind, the potential of *Bacillus* lipopeptides to step up as a candidate will be discussed next. Also to be discussed will be how their commercial application is hindered by lack of research to address challenges in cultivating and downstream processes.

2.4 *BACILLUS* LIPOPEPTIDES AS NOVEL BIOCONTROL AGENTS

Bacillus subtilis is an abundant bacterium commonly found in the water, air, soil and decomposing plant residue where they are responsible for nutrient cycling due to due to various biologically active enzymes and biologically active compounds they produce (US Environmental Protection Agency, 2015).

Soil populations are estimated at 10^6 per gram of soil and consist of a diverse number of rod shaped gram positive bacilli. The bacterium is equiped with a peritrichous flagella for mobility and produce an endospore that is highly resistant to unfavourable environmental conditions, heat and desiccation to ensure continuous presence in the soil (US Environmental Protection Agency, 2015).

The name *Bacillus subtilis* was given to all endospore forming bacilli until the development of more sophisticated DNA techniques that could better group species sharing the same apparent cellular morphology. The bacterium, *B amiloliquefaciens*, is an example of a culture once classified in the *B. subtilis* spectrum, but now classified as a separate *Bacillus* specie. Other closely related species to *B. subtilis* are *B. licheniformis* and *B. pumilus*. These were historically grouped together as *B. subtilis* based on similar characteristics of 368 strains (Priest, et al., 1988). These three species of "*Bacillus subtilis*" comprised 78% of the major gene cluster of the *Bacillus subtilis* genetic spectrum. With time and the

development of better analytical techniques this major cluster was separated and identified as four unique sub clusters of type species namely *B. subtilis, B. licheniformis, B. amyloliquefaciens* and *B. pumilus*, which are closely related (Sansinenea & Ortiz, 2011).

Bacillus subtilis. is classified as a GRAS organism (Generally Regarded As Safe) and labelled a Class 1 Contaminant Agent by the EPA and NIH and displays very low virulence with the capability of human infection almost non-existing. In being classified GRAS organisms, in combination with their highabundance in the soil, makes large scale production of lipopeptides utilising live *Bacillus* cultures safe in high concentrations. Very little risk is involved in adverse effects on society and the environments in case of accidental release into the environment, since the impact on soil concentrations should be negligible. Factories producing lipopeptides would also require less stringent process regulations around contamination, sterilising, limiting worker exposure to the bacteria, waste products etc. during culturing. This makes the *Bacillus* spp. an ideal candidate for research into developing novel biocontrol agents for large scale production.

2.4.1 Cyclic Lipopeptides

Once thought of as a mere lab contaminant, the *Bacillus* genus was found that its members have an average of 4–5% of the genome entirely devoted to antibiotic synthesis in the form of secondary metabolites and has been identified as a producer of over 21 structurally diverse biological antagonists, more specifically cyclic lipopeptides (Ongena & Jacques, 2008; Stein, 2005). These cyclic lipopeptides are classified as secondary metabolites (Chen, et al., 2008a; Alvarez, et al., 2012; Pretorius, et al., 2015) and are naturally synthesised in response to environmental stress, such as nutrient depletion, and allows the bacterium to compete for sources of food by killing off other organisms around it through antibacterial and antifungal activity (Pérez-García, et al., 2011). *Bacillus* secondary metabolites also assist in establishing synergetic relationships with plants and microbes, biofilm formation and helps with motility (Pérez-García, et al., 2011; Sansinenea & Ortiz, 2011).

The antifungals produced by *Bacillus* spp. mostly belong to cyclic lipopeptides (Alvarez, et al., 2012). Cyclic lipopeptides can be divided into three major families

which are currently being researched for their application in post-harvest biocontrol, namely the surfactins, fengycins and iturins (Figure 2-2) which display interesting physiological, biocidal and surface active properties.

The first major research paper of *Bacillus* lipopeptides that sparked scientists curiosity was with the discovery of a compound by Arima *et al.* in 1968, that exhibited surfactant like properties and reduced blood clotting. Today this compound is known as Surfactin.

Since then, scientists have investigated the versatile properties of *Bacillus* lipopeptides with focus in the pharmaceutical, cosmetic, environmental and food production fields. Commercially *Bacillus* could be exploited due to the wide variety of antibiotics, enzymes, proteases, amylases, inosine, ribosides, amino acids and speciality chemicals they produce. In recent years the focus however has shifted towards bioremediation and biocontrol.

Lipopeptides are synthesized non-ribosomally by *Bacillus* spp. via large multi-enzymes called non-ribosomal peptide synthetases or NRPSs (Stein, 2005; Finking & Marahiel, 2004) and leads to an broad collection of compounds amongst the synthesised lipopeptide structures with reference to type, amino acid sequence, cyclic moiety and branching of the acyl chain which all affect the microbial activity and effectiveness against the target specie(Figure 2-2). Surfactin and Iturin are both heptapeptides, 7 amino acid rings, with fatty acid chains of 12-17 carbons (Zhao, et al., 2011) while Fengycin is a decapeptide, 10 amino acid ring, with fatty acid chains of 14-18 carbons (Ongena & Jacques, 2008).

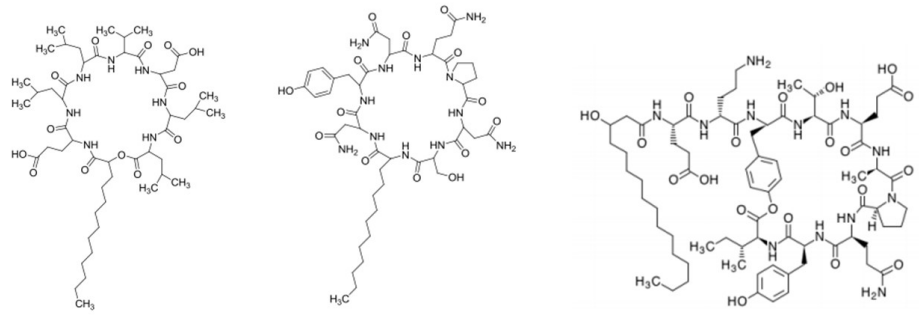

Figure 2-2 The three lipopeptide families, from left to right, Surfactin, Iturin and Fengycin. Molecules reprinted with permission (Pretorius, 2014)

The term “homolog” is applied to refer to different peptide varients within similar families amongst the diverse lipopeptides produced. Thus, 3 lipopeptides having the same amino peptide sequence of surfactin, but different fatty acid acyl chain lengths would be referred to as surfactin homolog 1 through 3. If the chain lengths are known, e.g. C13, C15, and C16, one could refer to the compounds as Surfactin homologs : Surfactin C13, Surfactin C15 and Surfactin C16.

As mentioned, the three main cyclic lipopeptide families of interest are Surfactins, Iturins and Fengycins. Within the surfactin family the main varients are esperin, lichenysin, pumilacidin and surfactin (Ongena & Jacques, 2008). The Iturin family contains variants identified as iturins (A, AL, and C), bacillomycin (D, F, L, LC) and mycosubtilin. The Fengycin family contains variants fengycin (A and B) and plipastatin (A and B). It is thus easy to see the diversity arising from three families which contain variants and display further diversity, homologs, due to peptides with varying fatty acid chain lengths. All of which will display varying degrees of activity against different organisms due to the unique chemical properties of each compound.

Showing high amhiphilicity and a tendency towards self-aggregation (Ishigami et al., 1995), hydrophobic interactions of the fatty acid chains with the cell membranes of target pathogens are thought to cause the formation of pores which result in the destabilization of the membrane and ultimately, cell lysis (Bernheim & Avigad, 1970). The precise mechanism of action of these lipopeptides, however, remains uncertain. This is especially true for Fengycins, for which no known mechanism has been identified (Ongena & Jacques, 2008). What is certain is the ability to destabilize the membrane either directly through detergent like properties, as is the case with Surfactin and Fengycin (Dehghan-Noudeh, et al., 2005; Deleu, et al., 2008; Seydlová, et al., 2011) or through Iturin’s displayed ability towards ion pore formation in target membranes (Aranda, et al., 2005). There are also documented synergetic effects among the cyclic lipopeptides (Maget-Dana, et al., 1992; Koumoutsi, et al., 2004; Romero, et al., 2007) where, for example, Surfactin, with no to low antifungal activity in combination with Fengycin has a higher activity than Fengycin alone. Research explaining this activity and mechanisms in details is lacking and a better understanding could help help speed up screening of lipopeptides, improve future product formulations and improve product performance.

Surfactin, isolated from *Bacillus subtilis* ATCC 21332, is currently the most potent surface active biomolecule known (Arima, et al., 1968) and displays strong antibacterial, antiviral, antimycoplastic and antitumor activity (Katz & Demain, 1977; Peypoux, et al., 1999; Nissen, et al., 1997; Zhao, et al., 2011). Antibiotics synthesised by *Bacillus* spp. such as difficidin, oxydifficidin, bacitracin, bacillin, and bacillomycin B (Parry et al., 1983) have revealed a broad range of antimicrobial activity towards a wide spectrum of both aerobic and anaerobic bacteria (Zimmerman et al., 1987). Fengycin and Iturin, generally restricted to *B. subtilis* (Bonmatin, et al., 2003) and *B. amyloliquefaciens* (Koumoutsi, et al., 2004), are effective inhibitors of a wide range of fungal diseases (Ongena & Jacques, 2008). In particular, *B. subtilis* strains have been demonstrated to display potent antifungal activity against phytopathogens in the grape and citrus industries, such as *Penicillium digitatum* (Leelasuphakul, et al., 2008; Yánez-Mendizábal, et al., 2011), *P. expansum* and *P. italicum* (Yánez-Mendizábal, et al., 2011), *Botrytis cinerea* (Touré, et al., 2004; Yánez-Mendizábal, et al., 2011), *Monilinia fructicola* (McKeen et al., 1986), , and *Rhizoctonia* (Loeffler et al., 1986).

Lipopeptides from *B. amyloliquefaciens,* have been identified as potential biocontrol agents that are specific against filamentous fungi (Koumoutsi, et al., 2004; Chen, et al., 2009; Vanittanakom, et al., 1986; Steller, et al., 1999; Loeffler, et al., 1986). Cultures of *Bacillus amyloliquefaciens* strains were found effective against *Sclerotinia sclerotonium* (white mould) (Alvarez, et al., 2012) and in this study it was found that Fengycin C16 and Iturin A C15 had strong efficacy towards the fungus. Notably, Surfactin C15 also displayed activity which led the team to conclude synergetic mechanisms are responsible for efficacy, which further the evidence for the presence of synergism. Other research supports Iturin A from *Bacillus amyloliquerfaciens* as inhibitor of citrus postharvest pathogenic fungi tested which include *Alternaria* and *Penicillium* (Arrebola, et al., 2010).

Iturins and Fengycin are also linked towards *Podosphaera fusca* inhibition (Romero, et al., 2007). Extracts of different lipopeptide homologs, each 1g/L, was prepared and tested on *Podosphaera fusca* in which Bacillomycin showed a 67% germination inhibition properties, followed by Fengycin, 53%, Iturin 42% and Surfactin 9.5% (Romero, et al., 2007). In the same study *Bacillus subtilis* strains displayed inhibition on the grape phytopathogen *Botrytis cinerea* up to 85-90% and 16 fold dilutions still inhibited 50% of cultures after 16 days post exposure.

Bacillus spp. could also specifically target *Aspergillus carbonaris* (Jiang, et al., 2014), *Aspergillus flavus* and *Aspergillus parasiticus* (Kimura and Hirano, 1988) which is responsible for the production of cancer causing agent ochratoxins during fungal decay of grapes and other fruits. For all of these post-harvest diseases the counter measure remains chemical pesticides (Mueller, et al., 2002; Romero, et al., 2007).

As mentioned, the main targets for lipopeptides are the cell membrane. Hydrophobic fatty acid tails of the lipopeptides insert into the fungal cell membrane causing it to either break apart as lipopeptide content in the membrane increase or they forms pores to leak cell content due to self-aggregation. The different levels regarding activity towards certain fungi in literature are explained by the sterol content of the fungi membranes under attack from lipopeptides. Sterol content of different fungi membranes is documented as a key parameter affecting antifungal activity (Avis & Bélanger, 2002) with low sterol content favouring lipopeptide activity and the buffering ability of cholesterol working against membrane destabilisation influencing antifungal properties (Romero, et al., 2007).

A major advantage lies in the non-specific target mechanism regarding membrane destabilisation. Through hydrophobic interactions of the lipopeptides with the cell membranes, this mechanism provides protection against resistance development unlike those seen in chemical pesticides (Glare, et al., 2012; Mandal, et al., 2013) and promises an effective control with broad-spectrum activity properties.

Apart from its antimicrobial (fungi, bacteria, viruses, oomycetes, yeasts) and physiological abilities to supress tumour growth, counter inflammation and bioremediation, the genus has also been shown to be capable of eradicating the mosquito larvae, *Anophelis culicifacies*, which isnresponsible for transmitting malaria in central India (Gupta & Vyas, 1989) and can also be effective at stimulating the plant's defences against other nematodes and insects.

Lipopeptides also have the advantage over synthetic pesticides in being biodegradable, exhibiting a very low toxicity, are regarded environmentally benign, have no impact towards beneficial organisms in the niche they are applied and have greater activity at lower doses that those needed in chemical applications. (Desai & Banat, 1997; Thakore, 2006; Banat, et al., 2010). Lipopeptides are resistant to extreme pH and salanity (Banat, et al., 2010) and pose a reduced risk to mammals

and birds due to not bioaccumulating (Thakore, 2006). Research into chemical stability found lipopeptides are stable at 50-100°C tested and resistant to enzymatic degradation from pronase and proteinase K (Romero, et al., 2007).

Though displaying notable benefits over their synthetic counterparts, the major drawback keeping biocontrol products from being developed is high production cost and lack of research into optimised downstream processing methods for isolation and purification that make them economically viable (Banat, et al., 2010).

2.4.2 Applications of *Bacillus* Lipopeptides

Most of the research and field application focusses on the application of the live organism on the plant and/or fruit which would then produce the antifungals to protect the product, but research done by Yánez-Mendizábal (2011) found that cell-free supernatant extracts performed vastly better, 89-100% inhibition, than the application of the organism itself, 40-73%.

These results can be explained by the impact changes in the environment have on lipopeptide production compared to finely controlled parameters in the lab or in industrial manufacturing. A change in the environmental parameters such as temperature, pH, moisture, nutrients etc. have a significant impact on the organism's ability to produce lipopeptides. This is supported in research done by Ohno et al. (1995) which found that changes in temperature change the profile of different lipopeptide products being produced. Application of live organisms rarely resulted in activity more than a few days post application (Glare, et al., 2012)

Using live organisms in the formulation of biocontrol products puts the product up for strict regulations by government with quarantine measures and the need for toxicology tests of the product (Glare, et al., 2012).

All these drawbacks can be overcome by using a cell-free formulation produced in a controlled environment, as will be discussed and examined in this study.

2.4.3 Lipopeptide Production, Limitations and Cost

One of the major challenges facing scientists in the advancement of biopesticides to replace chemical pesticides lies in the development of financially competitive processes for production in comparison to relatively "cheap" chemical synthesis.

Fermentation of *Bacillus* lipopeptides is more expensive than chemical synthesis of current fungicides and current formulations and process strategies are kept as trade secrets by the companies developing them (Glare, et al., 2012). This could be attributed to the fact that around 10 years on average is spent by these companies from screening organisms to isolating their product for development (Glare, et al., 2012) where the steps include screening each strain's resistance to extreme environments, projecting cost for mass production, environmental compatibility and so forth.

Surfactin has been known to be haemolytic (Dehghan-Noudeh, et al., 2005) so ideally, if it posed a health hazard, one would ideally strive to remove it from the final product, which makes downstream processing is costly, if current methods are used. It is however unlikely that Surfactin could pose a severe risk since Seydlová, et al. (2011) claims the haemolytic effect of surfactin causing 50% haemolysis to be between 30-300µM and no toxic effect was observed in mice treated with Surfactin up to 2500mg/kg.

None the less, the need for downstream processes to enrich products increases the total production cost with chemical isolation during downstream processing being the most costly (Keller, et al., 2001). Up to 60% of process cost is estimated to lie within downstream isolation techniques (Desai & Banat, 1997). Due to the high cost, the product would need fewer steps to remain financially feasible from the current lack of optimised production processes in this point of time. Also, most processes in development remain on lab scale (Keller, et al., 2001) and thus considerable research is still needed for scaling up these experiments.

The proposal for development steps towards optimising lipopeptide production is summarised in Figure 2-3. The first step is to screen for viable cultures and the development of over-producer strains. No data could be found pertaining to existing strains meeting this requirement.

The second crucial focus point is the development of optimised culturing parameters where the need for expensive substrates and micronutrients could

possibly be replaced by cheaper waste or renewable sources of nutrients. Importantly, oxygen transfer has been identified as a limiting factor in production (Sheppard & Cooper, 1990), findings which are supported by Pretorius et al. (2015), which focussed on culture media and process optimisation and also concluded new solutions to overcome oxygen depletion during culturing needs to be investigated.

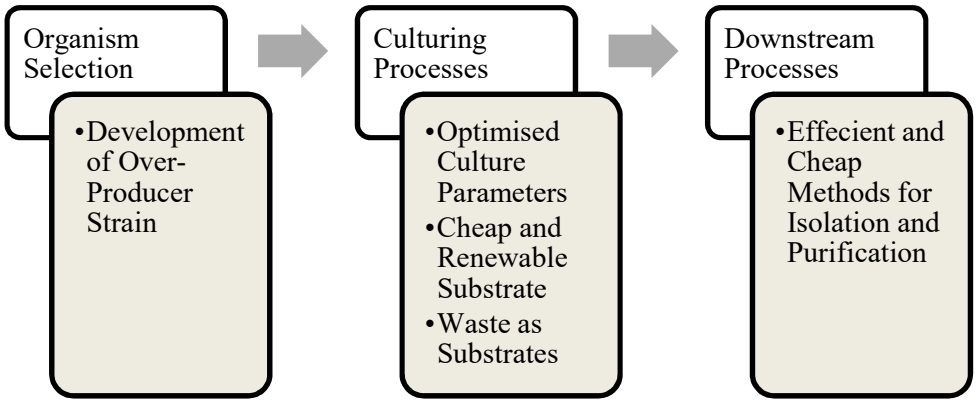

Figure 2-3 Optimisation strategies for cost reduction. Adapted from Banat et al. (2010)

Looking at current downstream methodologies in literature towards optimisation strategies, Desai and Banat (1997) summarises the downstream processing techniques used for lab scale isolation of lipopeptides. These are ammonium sulphate precipitation, acetone precipitation, acid precipitation, solvent extraction, crystallisation, centrifugation, foam separation, filtration and ultrafiltration. Notably of all these techniques acid precipitation followed by solvent extraction remains the norm for Surfactins, Fengycins and Iturins (Ohno, et al., 1992; Chen, et al., 2008a)

To investigate the isolation strategies for lipopeptides downstream, the methods occurring in research the most are discussed next.

2.4.3.1 Acid Precipitation

Lipopeptides undergoing pH adjustment have a bulk precipitation occurring at <pH 2 (Romero, et al., 2007). Research using 6M HCl, pH 2 (Vater, et al., 2002;

Sivapathasekaran, et al., 2009), 3M HCl, pH2 with a yield of 547 mg/L (Kim, et al., 2004), and 1M HCl, pH 4 (Chen, et al., 2008a) are documented.

Following this route of isolation, through acid precipitation, results in a lack of purity, with a total of 55% lipopeptide purity documented (Chen, et al., 2008a; Chen, et al., 2007). This is attributed to other media proteins and culture constitutes co-precipitating.

2.4.3.2 Solvent Extration

Solvent Extraction is another method for isolating lipopeptides due to the presence of amphipathic nature of the molecule and the hydrophilic chain present. The downside is the use of toxic solvents in some cases and also the cost involved using these solvents downstream.

Methods using chloroform-methanol, methanol, dichloromethanol-methanol, butanol, ethyl acetate, pentane, hexane, ether (Desai & Banat, 1997) n-hexane, ethyl acetate (Chen & Juang, 2008) and n-butanol (Yazgan, et al., 2001; Romero, et al., 2007; Arrebola, et al., 2010) have been documented as extraction solvents.

It is also possible to combine solvent extraction with acid precipitation such as dichloromethane followed by precipitation with 6M HCl, pH 2 (Dehghan-Noudeh, et al., 2005).

2.4.3.3 Ultra Filtration

To reduce the use of solvents, mechanical processes such as ultrafiltration and nanofiltration are being employed on a lab scale to research their effectiveness for use in larger industrial applications to isolate lipopeptides.

Ultrafiltration boosts the recovery to 72% with a marked 87% purity of surfactin, compared to 55% purity with acid precipitation alone (Chen, et al., 2007). When combining salting out with ultrafiltration, >81% is achieved (Chen, et al., 2008a).

The highest known isolation results is up to 95% using ultrafiltration coupled to ion-exchange chromatography (Chen, et al., 2008b) and 90-98% recovery of surfactin by two stage filtration (Isa, et al., 2007).

Filters with an exclusion limit of 3kDa retained Fengycin (Romero, et al., 2007) and was recommended for this route.

2.4.3.4 Other Isolation Approaches

Sivapathasekaran, et al. (2009) followed the development of a HPLC method for lipopeptide separation from media, followed by fraction collection, acid precipitation at pH 2 and dissolving the precipitates in methanol. This method would be very expensive to employ on a large scale due to the many steps involved, but offers good separation efficiency among the lipopeptide families.

An interesting method for direct isolation of lipopeptides was employed using a special culture flask with a "funnel" to concentrate the top layer of a media culture (Glazyrina, et al., 2008). When the flask was tilted to the side, the top layer was forced into the narrow neck, the top layer isolated and removed and the lipopeptides extracted with methanol (Figure 2-4).

Similarly, a possible cost effective method could simply employ foam isolation during culturing. Foam isolation (Davis, et al., 2001) recovered 1.22 – 1.67 g/L surfactin which translated in a 60% recovery.

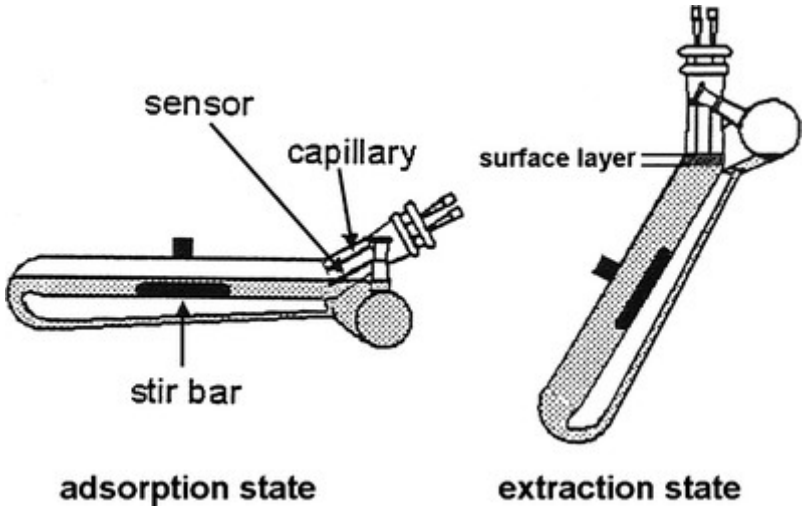

Figure 2-4 Diagram of apparatus for biosurfactant isolation developed by Glazyrina, et al., 2008.

Another research group went further in custom reactor design and employed pertraction, a form of liquid-liquid extraction, to separate surfactin. (Dimitrov, et al., 2008) The elaborate setup uses rotating disks in a three phase liquid membrane setup using n-heptane, 1-octanol and combinations thereof. The method documented 83.44% efficiency for isolating surfactin with n-Heptane and 100% with 1-Octanol at pH5.5.

2.4.4 Media Composition and Strain Selection for Culture Experiments

In a paralleled study to this thesis, Pretorius et al. (2015) tested four *Bacillus* spp. strains on an optimised media in a fully automated bioreactor. The study presented here will focus on the growth of these four strains, but in shake flaks.

In short, the strains chosen for the study presented here were *B. amyloliquefaciens* DSM 23177*, B. subtilis ATCC 21332, B. licheniformis* DSM 13 and *B. spizizenii* DSM 347 and were selected based on the broad range of documented lipopeptides produced (Arguelles-Arias, et al., 2009; Chen, et al., 2009; Banat, et al., 2010). Media was developed using the formulation from Kim et al. (1997) as base a media, which according to literature achieved 4.5 g/L surfactin on glucose (Pretorius, 2014), and adapting the microelements using research findings from Wei et al. (2007). The final media composition can be found in the methodology section

Of the four strains cultured using controlled bioreactor studies, Pretorius et al. (2015) identified *B. amyloliquefaciens* DSM 23177 as the best candidate. This was due to high antifungal production coupled with low surfactin production and the strain displayed adequate inhibition towards *Botrytis cinerea* DSM 877.

2.5 SUMMARY

Through the indiscriminate use of vast quantities of chemical pesticides the world has seen damage inflicted on the environment and an increase observed in resistance development in phytopathogens that cause severe post-harvest losses. New technologies are needed to break the cycle of resistance development and offer environmentally benign control with fewer drawbacks from use.

In this chapter we have investigated the potential for biopesticides as the next generation of pesticides in food production and post-harvest protection of crops.

Through their non-specific action they oppose resistance development in phytopathogens treated and they are preferred to synthetic pesticides due to their

low toxicity compared to synthetic pesticides (which usually are hazardous chemicals), broad spectrum efficacy, environmentally compatible, biodegradability, lack of toxicity towards beneficial microorganisms, mammals and insects and require lower effective doses. The product is stable at various environmental conditions such as pH and temperature and the *Bacillus* bacterium responsible for their production is labelled a GRAS organism by government which makes them ideal candidates for use in large scale production.

In the last 5 decades research on *Bacillus* lipopeptides has seen significant growth towards the application and general understanding of these versatile compounds, but their application in the field is hampered by high production cost and lack of optimised processes. Thus, reseach is currently focused on developing competitive fermentation and isolation parameters for mass production.

Of all the lipopeptides produced, the Fengycin and Iturin families have been identified as key components linked to antifungal activity against a broad spectrum of phytopathogens. This study is focussed on *Bacillus* antifungal lipopeptides on post-havest phytopathogens in the South African Table Grape Industry to determine their potential application as a biocontrol agent.

Thus, with the antifungal lipopeptides as target for isolation and testing in mind, the following major points of interest for investigation are highlighted from the literature:

- None of the studies addresses the question of which fraction of the culture media contains which quantity of lipopeptides, i.e. the supernatant and foam contain documented concentrations of the product, but what about the cellular matrix itself? And when cells are removed via centrifugation, could undetected precipitates of the product contain lipopeptides? Answers to these could help improve process design to increase yield.

- No documented research could be found documenting the different lipopeptide homologs produced by each of the four strains, *B. amyloliquefaciens* DSM 23177*, B. subtilis* ATCC 21332*, B. licheniformis* DSM 13 and *B. spizizenii* DSM 347, and during which stage of growth they are produced.

- Which of the lipopeptides produced by *B. amyloliquefaciens DSM23177, B. subtilis ATCC 21332, B. licheniformis DSM 13* and *B. spizizenii DSM 347* are responsible for any antifungal activity?

- In most downstream studies the focus lies on total isolation of lipopeptides and not separation efficiency among lipopetides. It is thus needed to determine the separation efficiency of the different methods to separate the three lipopeptide families from each other within a sample. Answers to this parameter could shed light on new applications of the method or combination of methods specific to antifungal isolation.

- Minimum inhibition concentrations for each of the lipopeptide homologs, which inform on the concentrations needed in formulations, are missing from literature.

Chapter 3

METHODOLOGY

The aim of the project was to determine the prospect of biologically synthesised lipopeptides to be used as novel biocontrol agents against phytopathogens responsible for postharvest disease.

As such, *Bacillus* spp. cultures were screened for lipopeptide production and the crude supernatant samples of lipopeptides mixtures tested for antimicrobial activity. Phytopathogenic fungi infecting South African grapes were isolated from postharvest table grapes, purified and identified through genetic sequencing in conjunction with microscopic identification. Isolates of fungi were used in antimicrobial studies. The bacterial cultures that showed high antagonistic activity were further assessed towards isolation strategies for lipopeptide present, crude purification thereof and calculating minimum inhibition concentration (MIC) values which could inform on future process development and optimisation.

3.1 CULTURING ASEPTIC PROTOCOLS

All preparations for experimental procedures and cell culturing were performed in a sterile environment in a laminar flow cabinet (ClearFlow, USA) with aseptic techniques practised throughout. Equipment was heat sterilised via autoclaving (Quick Clave, Gemmy Ind. Co., USA) unless stated otherwise.

3.2 CULTURES AND CELL BANK MAINTENANCE

3.2.1 Bacillus Stock Cultures

Four different strains of lipopeptide producing bacteria, *Bacillus amyloliquefaciens* DSM 23117, *B. licheniformis* DSM 13, *B. subtilis* ATCC 21332 and *B. subtilis spizizini* DSM 347 were obtained as freeze dried cultures from type culture banks; The American Type Culture Collection, ATCC, America and the Deutsche Sammlung von Mikroorganismen und Zelkulturen, DMSZ, Germany.

3.2.2 Phytopathogen Stock Cultures

Additional to the phytopathogens isolated, *P. digitatum* DSM 2751 and *B. cinnerea* DSM 877 were obtained from type culture banks. Potato dextrose agar (PDA) plates containing 100mg/L Ampicillin were routinely used during culturing under aseptic conditions. All plates were stored at 4°C until use.

3.2.3 Cell Revival and Long Term Storage Protocols

The strains were revived from freezer stocks by addition of 1 ml nutrient broth media or malt extract media to the applicable freeze dried culture, followed by 30 mins of incubation at 30 °C for bacteria and 26 °C for fungi in a shaker-incubator (Labcon FSIM-SPO24, Labcon, USA). Nutrient agar (NA) and PDA plates were streaked with 100 µL from each relevant culture tube and incubated for 24 h and then stored at 4 °C until use. Slower growing fungi cultures where grown for 3-7 days before storage.

For medium term storage the *Bacillus* spp. cultures were maintained on NA slants and fungi on PDA slants at 4 °C. These culture stocks were subjected to sub-culturing every two months to keep the culture stocks viable.

For long term storage, backup cultures where stored as glycerol stocks and freeze-dried pellets. Glycerol stocks were prepared from active growing shake flask cultures in the exponential phase by 1:1 addition of pre-sterilised 80% glycerol in 0.85% saline solution. After 30 minutes of incubation 1 ml of the glycerol culture solution was transferred to a gamma sterilized 2ml Cryotube (Lasec, South Africa), flash-freezed in liquid nitrogen and immediately stored at -20 °C for up to a year.

Freeze dried cultures where prepared by pelleting active growing shake flask cultures in a 2ml Eppendorf tube at 14 000 RPM (Minispin plus, Eppendorf, Germany), followed by addition of cell pellets into one tube to concentrate cells. To protect cells from the freeze drying proses, 1ml lyoprotectant was added. Lyoprotectant was prepared consisting of 0.75g Tryptic Soya Broth (TSB), 10g sucrose and 5g Bovine Serum Albumin (BSA) in 100ml dH_2O and filter sterilised through a 0.22 micron nylon filter. Cryotubes where labelled with the appropriate identification and 0.5ml of the culture mixture was transferred to a 2ml cryotube and subjected to lyophilizing overnight. Freeze dried pellets where stored at 4 °C.

3.3 *BACILLUS* SPP. SHAKE FLASK EXPERIMENTS

3.3.1 Media Composition

Different media composition recipes were obtained from literature and two with the highest reported yield used as a guide to design an optimised media.

Controlled bioreactor trails were performed in a 1L reactor (New Brunswick Scientific) with pH and temperature control using Kim et al. media as discussed in shake flask experiments below.

Shake flask experiments were carried out to determine the best pH buffer and nitrate concentration (Media A-H, figure introduction) with media A being from Kim et al, 4% (w/v) glucose, 170 mM NH_4Cl, 60 mM K_2HPO4, 12.5 mM NaH_2PO_4, 2 mM $MgSO_4$, 0.3 mM $MnSO_4$ and 0.05% (w/v) yeast extract. Kim et al media, media A was also tested in a fully controlled bioreactor (New Brunswick Scientific, USA) equipped with pH control using 1 M NaOH.

Media B comprised of 4% (w/v) glucose, 50 mM NH_4NO_3, 1.26 mM K_2HPO4, 2.04 mM $KH2PO_4$, 2.4 mM $MgSO_4$, 0.01 mM $MnSO_4$, 0.008 mM $FeSO_4$, 7 µM $CaCl_2$ and 0.05% (w/v) yeast extract. Media C was the same as media B, but excluded yeast extract.

Media D and E contained 4% (w/v) glucose, 50 mM NH_4NO_3, 6.1 mM K_2HPO4, 9.9 mM $KH2PO_4$, 2.4 mM $MgSO_4$, 0.01 mM $MnSO_4$, 0.008 mM $FeSO_4$, 7 µM $CaCl_2$ and only media D included 0.05% (w/v) yeast extract.

Experiments conducted with Media F contained 4% (w/v) glucose, 50 mM NH_4NO3, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, 2.4 mM $MgSO_4.H_2O$, 0.01 mM $MnSO_4.H_2O$, 0.008 mM g/L $FeSO_4.7H_2O$, 7 µM $CaCl_2.4H_2O$ and 0.05% (w/v) yeast extract.

The effect of increased nitrate availability on lipopeptide production was investigated. Media G and H both were derived from media F, but contained double the amount of NH_4NO_3, 100 mM, while media H had no yeast extract.

3.3.2 *Bacillus* Lipopeptide Screening

All experiments, unless otherwise stated, were done in triplicate. Shake flask experiments were carried out due to their ease of operation and usefulness in large quantity screening in parallel.

Bacillus spp. strains were pre-cultured in 100 ml Erlenmeyer flasks containing 50 ml of culture media and was inoculated with a single colony from the *Bacillus* plates and incubated for 16 h. From this culture 10 ml of was transferred to 200 ml nutrient solution. Culturing was performed in triplicate in 500 ml bevelled shake flasks at 30°C in an orbital shaker incubator at 150 RPM. Sampling was done by removing 2ml every 24 hours for 7 days.

3.3.3 Screening Media Fractions for Lipopeptides

Media A was sampled and fractioned into foam, precipitate, cells and supernatant.

Foam was collected and left to settle into liquid. The volume was adjusted to the original volume using saline water to relate the isolated concentration to the gram per volume of culture.

The remaining cells and precipitate was centrifuged at 14 000 RPM (Minispin plus, Eppendorf, Germany) and the supernatant removed and kept.

The remaining cells and precipitate was separated by adding a small amount of saline water to the cells and precipitate and bringing the pH up to neutral using 0.1M NaOH. The volume was adjusted to the original volume sampled and centrifuged immediately at 14 000RPM and separated into cells and “precipitate” in solution. The supernatant was kept and the cells suspended in saline water equal to the sample volume.

The cell sample was sonicated for 3 minutes to break open the cells.

All four samples were analysed using HPLC in section 3.5.3.

3.4 CELL GROWTH ANALYSIS

3.4.1 Cell Dry Weight

The cell concentration of each sample was quantified as cell dry weight (CDW), calculated from a pre-constructed CDW *vs*. optical density at 620 nm (OD_{620}) standard curve from *Bacillus amyloliquefaciens*. The curve was constructed by measuring the OD_{620} of a cell solution (cells resuspended in 0.85% (w/v) NaCl physiological saline solution) and filtering 5 mL of sample through a pre-weighed filter paper (0.22 µm, pre-dried for 24hrs under vacuum at 80°C). The paper was weighed after drying, 24hrs under vacuum at 80ºC, and the CDW calculated per litre corresponding to each OD_{620} reading.

The OD_{620} during experiments was measured by centrifuging 1 ml samples of the culture at 14 000 RPM for 5 min (Eppendorf Mini-Spin Plus). The supernatant was removed and the pellet resuspended in 1 ml physiological saline solution. The OD_{620} was recorded with a spectrophotometer (Varian Cary 1E, Agilent Technologies, USA), using physiological saline as a blank, and converted to CDW using the equation CDW = 0.354 x OD (R^2=0.9944) obtained from the CDW standard curve.

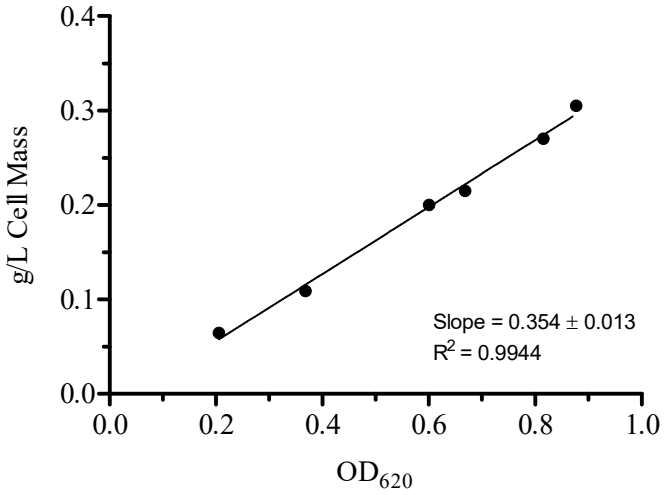

Figure 3-1 CDW vs. OD_{620} calibration curve constructed from *Bacillus amyloliquefaciens* for cell density determination.

3.4.2 Gram Staining for Validation

Actively growing experiments were continuously tested for bacterial cross-contamination via Gram staining. Cells of samples from culture media were smeared on a clean microscope slide and allowed to air dry. The slides were treated with crystal violet for 20 seconds followed by removal of the crystal violet by tilting the slide. Grams iodine was then added to the slides and left for 1 minute. The slides were then tilted again to remove the iodine and carefully washed with 75% Ethanol. To check for gram negative bacteria Safranin was added to the slides for a final 20 sec. The solution was removed by tilting the slides and left to dry. Dried slides were inspected under 1000x magnification using a light microscope (AxioStar Plus, Zeiss, Germany).

3.4.3 Sporulation

During culturing the growth media was tested for spores or cells containing internal spores which indicate stress or the early start of stationary phase by staining spores. Samples taken from culture media during various time periods in culturing were smeared on a clean microscope slide and allowed to air dry. Malachite green was added to the slides and covered with tissue paper for 5 minutes. The solution was removed with water and dried slides inspected under 1000x magnification using a light microscope. Using this method, sporulation could be observed that indicated the start of stationary phase and expected antifungal production.

3.5 SUBSTRATE AND PRODUCT ANALYSIS

3.5.1 Glucose Concentration

Standard curves were constructed using dilutions prepared from pure glucose (Merck, Germany) ranging from 0.15 – 10 g/L.

Samples were prepared from media samples taken from shake flask culturing. Samples were filtered through 0.22 µm filters prior to quantification via HPLC (SpectraSYSTEM, Thermo Separations Product™, USA). Injection of 30µL were separated on an Aminex HPX-87H column (300 x 7.8 mm, 9µm, Biorad, USA) through isocratic separation using 0.005 mM H_2SO_4 as mobile phase. The flow rate was adjusted to 0.6 ml/min and Glucose detection was accomplished with the use a refractive index detector (Shodex RI-101, Dionex, USA).

Calibration data can be found in Addendum A.

3.5.2 Nitrate and Sulphate Concentration

Nitrate and Sulphate concentrations for media samples were determined using an IonStar A300 column (100x4.6mm, Phenomenex, USA) on a Dionex 4500i chromatography system equipped with an AMMS II ion suppression module and conductivity detection at ambient temperature.

Isocratic separations were performed with eluent A containing 1.80 mM Na_2CO_3 and 1.70 mM $NaHCO_3$ in ultrapure water (Merck Millipore System, Germany) at 1.5 ml/min. The ion suppression regenerant solution, 0.025 mM H_2SO_4, was set at 3mL/min and 20 µL injections were made on the column.

Pre-dried $NaNO_3$ and Na_2SO_4 was used to prepare a serial dilution standard range from 5-200 mg/L (ppm) NO_3^- and SO_4^{2-}.

Calibration data can be found in Addendum A.

3.5.3 Lipopeptide Characterization and Quantitation

Antifungal lipopeptides, iturin, fengycin and surfactin concentrations were measured quantitatively by reverse phase HPLC (RP-HPLC). Samples were prepared by centrifugation of a 1 ml culture at 14 000 RPM for 5 min. To quantify the supernatant concentrations, the supernatant was added to an aqueous solution of 80% acetonitrile (v/v) in a 1:1 ratio of sample to acetonitrile. All samples were

filtered by means of a 0.22µm syringe filter (Merck Millipore, Germany) before HPLC analysis.

Quantification of lipopeptide production was achieved by RP-HPLC on a Dionex Ultimate 3000 System with Ultimate 3000 UV detector by loading 100 µl of each HPLC sample on a Luna C18 column (150 x 4.6 mm, Phenomenex, USA). The column was eluted with mobile phase A, 0.1% (v/v) trifluoroacetic acid (Fluka®) in water, and mobile phase B, 0.1% (v/v) trifluoroacetic acid in acetonitrile (Chromasolv, Sigma-Aldrich®), over a acetonitrile gradient, 35% - 80%, over 70 minutes. The flow rate was 1ml/min and the detector was set at 210nm for lipopeptide detection.

Surfactin standards were prepared for quantification from pure surfactin (Sigma-Aldrich®) by adding 10mg to 1ml methanol (i.e. 10g/L) and performing a serial dilution ranging from 0.15 g/L - 5 g/L. Iturin standards were prepared from pure Iturin (Sigma-Aldrich). In the absence of a Fengycin standard, Fengycin was quantified using the calibration data from related research by S Mazibuko and KG Clarke, equation 1. (data unpublished).

Equation 1 $$Concentration = AU \times (7.09 \times 10^{-4})$$

Calibration data can be found in Addendum A.

3.6 PHYTOPATHOGEN ISOLATION

South African table grapes infected with various phytopathogens were supplied from farms in the regions of the Hex River Valley and from the Swartland by the South-African Table Grape Industry (SATGI). Tissue samples of infected fruit were taken aseptically from the berry skins, pedicle, brush and internal seeds and purified, in triplicate, by a sequential sub-culturing procedure on malt extract agar (MEA) and potato dextrose agar (PDA) plates containing 100mg/L ampicillin to prevent bacterial contamination and nutrient agar (NA) containing no antimicrobials. To do this, single colonies on each starter plate displaying different colony colour, shape, size and textures were picked up aseptically in the laminar flow cabinet with a flame sterilised inoculation loop and plated on fresh agar plates until pure cultures were achieved. All plates were subjected to a minimum of 5 rounds of sub-culturing to achieve pure cultures.

Additionally fungi cultures, *P. digitatum* DSM 2751 and *B. cinnerea* DSM 877 from culture banks were also included for their involvement in post-harvest disease in the citrus and grape industries respectively.

Plates were inoculated and incubated right side up for 24 hours at 26°C after which plates were turned upside down and incubated for an additional 72 hours or until identifiable fungal colonies formed.

All plates were stored at 4°C until use.

3.7 PHYTOPATHOGEN IDENTIFICATION

Purified phytopathogen cultures, isolated from the field were subjected to genetic sequencing and microscopic analysis for identification. By combining both morphological studies and genetic sequence alignments, the fungi genera could be identified.

Morphology studies were done on the basis of plate colony colour, shape, and texture and growth patterns to determine the macro features in combination with microscope analysis to determine microscopic structures such as hyphae appearance, the presence of fruiting bodies, structures and spores.

3.7.1 Genetic Sequencing

Fungi cultures were selected for DNA analysis and sent for sequencing at the Central Analytical Facility, CAF, Stellenbosch University where fungi from actively growing plates were subjected to DNA extraction, PCR amplification and Sanger sequencing. Genetic material was isolated from fungal mass based on methods by Justesen et al. (2002).

The extracted DNA was subjected to PCR amplification prior to sequencing. This was achieved through the use of both the universal forward and reverse primers, ITS4-(TCCTCCGCTTATTGATATGC) and ITS5-(GGAAGTAAAAGTCGTAACAAGG), corresponding to the ITS1 and ITS2 (internal transcribed spacers) regions. These regions are highly variable among the fungi genera and thus an ideal tool for identification (Schoch, et al., 2012).

Setup for PCR was done by adding 2 µl of 10ng/µl DNA extract to 10µl 2X KAPA 2G Robust Hotstart Readymix (KAPA BIOsystems, USA), 4 µl 5X KAPA Enhancer 1, 0.4 µl ITS 4 (10 µM) an 0.4 µl ITS 5 (10 µM) primers and 3.2 miliQ H_2O to a final volume of 20 µl. Amplification was initiated (Veriti® 96 well Thermal cycler, Life Tech™, USA) by initial denaturing of DNA fragments at 95°C followed by 30 cylces of denaturing at 95°C (30sec), annealing of primers at 51°C (30sec) and chain elongation at 72°C (45 sec).

Successfully amplified DNA material was sequenced (3730 XL DNA Analyzer, Life Tech™, USA) and the ITS 4 and ITS 5 fragments aligned through an online nucleotide BLAST (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov).

3.7.2 Fungal Colony Microscopic Analysis

Wet slides were prepared from actively growing cultures and observed under 40x magnification (AxioStar Plus, Zeiss). Small samples of hyphae from each plate were taken and suspended from a slide using a drop of water using the hanging drop method. Identification was based on hyphae structures present and morphology of fruiting bodies.

3.8 LIPOPEPTIDE BIOLOGICAL ACTIVITY ASSAYS

3.8.1 Radial Diffusion Assays

Anti-fungal activity was tested using PDA plates with four equidistant 5mm circular wells cut into the agar. A plug of the target fungus was placed in the centre of the plate and incubated for 24 hours at 26°C. Supernatant, 50 µl, from four *Bacillus* spp. cultures was loaded into each of the wells with water as a control in one well. The plates were incubated right side up at 26°C for 24 hours and the resulting diameter with no fungal growth was recorded after 3-7 days with a metal ruler when clear halos were visible.

3.8.2 Haemolytic Activity

The isolated lipopeptide samples were screened for haemolytic activity using blood agar plates consisting of nutrient agar supplemented with 5% (v/v) human blood (Western Province Blood Transfusion Service, South Africa). Testing was performed by loading 50 µl of supernatant from *Bacillus* spp. cultures into wells and incubating at 30°C for 24 hours. The diameter of the resulting halos were measured and used to compare haemolytic activity among the different strains.

3.8.3 Bioautographic Assays

To isolate lipopeptides responsible for fungal inhibition, bioautograpic assays were perfomed on TLC plates of separated lipopeptide compounds (see section 3.9.4) by overlaying each plate with PDA agar inoculated with 1% (v/v) of a *B.cinnerea* spore solution in 0.85% physiological saline. The plates were incubated at room temperature for 4 days and inspected for inhibition.

The retention factor value (R_f) for spots displaying inhibition were calculated using the equation below:

Equation 2 $$R_f = \frac{\text{Distance of compound from origin}}{\text{Distance of solvent front from origin}}$$

3.8.4 Minimum Inhibition Concentration Assays

To determine the effective dose of the antifungal lipopeptide on fungal pathogens, minimum inhibition concentration assays (MIC) were performed using serial dilutions of lipopeptides of known composition in 96 well microtiter plates ranging from 1 g/L to 0.04 g/L.

Fungal spores isolated from pure *B. cinnerea* were suspended in a potato dextrose broth (PDB) solution and added to the wells containing the lipopeptide dilutions to a 10% (v/v) concentration.

The titre plate was incubated at 26°C for four days and the wells inspected for growth. The wells between growth and no growth were determined as the MIC.

3.9 RECOVERY AND PURIFICATION STRATEGIES

3.9.1 pH Precipitation

Crude media was isolated from 24 hour old *Bacillus* spp. cultures in late exponential phase, the pH adjusted to pH 6.0-7.0 with 1M NaOH/HCl and centrifuged at 6000 RPM for 10 minutes to remove solids.

The supernatant was removed, a 1 mL sample taken for recovery calculations and the remaining split into 30 mL fractions in 50 mL falcon tubes. These tubes were used for precipitation and centrifugation steps. Using an automated Hanna HI902 titrator (Hanna Instruments, South Africa) set up with 0.1M HCl titration solution, the pH of each fraction was lowered in increments of 1 pH points from pH 6 to pH 1.

The pH was automatically monitored by the instrument, but to ensure accuracy of the probe (due to the presence of large protein quantities that could foul the probe membrane) the pH was also confirmed at the end of each titration using universal pH strips (1.09535.0001, Merck Millipore, Germany). The probe was cleaned with probe cleaning solution, 0.5M HCl for 10mins.

After each titration was completed the fraction was left at room temperature on the bench for an additional 30 minutes to ensure complete precipitation and then centrifuged at 6000 RPM for 30 minutes at room temperature (CN-2060, MRC, South Africa). The supernatant was transferred to a clean falcon tube, the precipitated pellet resuspended in 1 mL of 50% (v/v) acetonitrile:water and mixed thoroughly by means of gentle vortexing. Finally, the samples were filtered through 0.22 µm syringe filters into HPLC vials for lipopeptide quantitation using RP-HPLC.

3.9.2 Ammonium Sulphate Precipitation

Salt precipitation of lipopeptides and media proteins was carried out by stepwise volume addition of a saturated solution of Ammonium Sulphate, $(NH_4)_2SO_4$.

To prepare a saturated solution of Ammonium Sulphate, 76.6 g of $(NH_4)_2SO_4$ was dissolved in 100 mL of distilled water at 30°C. Any un-dissolved crystals were left to settle to the bottom and insured the solution stayed saturated. The solution was stored at room temperature until use.

Liquid aliquots of the saturated $(NH_4)_2SO_4$ solution were removed without disturbing the crystals at the bottle and filtered through a 0.22 µm filter just before precipitation experiments.

Cell free supernatant rich in lipopeptides, derived from centrifugation of 24 hour old cultures of *Bacillus amyloliquefaciens,* was concentrated by evaporating 1 L of the crude supernatant under vacuum at 40°C. The resulting crude concentrate was frozen and kept at -20°C until use, due to the significant amounts of sugar and nutrients still present.

Before precipitation experiments, the crude concentrate was thawed and redissolved in 10 mL of 0.22 µm filtered distilled water to give a concentrated lipopeptide solution. Any undissolved particles were removed via centrifugation at 6 000 RPM for 10 minutes and the concentrate split into two 5 mL aliquots.

Using a 25 mL burette, the 5ml aliquots of concentrated lipopeptide solution in 50 mL falcon tubes were stepwise precipitated through addition of saturated $(NH_4)_2SO_4$ as found in Table 3-1.

After each addition of $(NH_4)_2SO_4$ the 50 mL tubes were gently mixed and centrifuged at 6000 RPM for 30 minutes. The supernatant was decanted into a new 50 mL tube, a 500 µL sample of the fraction removed for SO_4^{2-} analysis, and the remaining supernatant kept for the next addition of $(NH_4)_2SO_4$.

Table 3-1. Salt precipitation table of volume of saturated ammonium sulphate solution to add to each sample to reach the desired concentration as a percentage of saturation (For mulitplication factors, see Addendum B).

Fraction	*Sample Volume*	*Desired Conc. %*	*Multiplication Factor*	*Vol. $(NH_4)_2SO_4$ Addition**
0	5.000	10	0.111	0.555
1	5.055	20	0.125	0.632
2	5.187	30	0.143	0.742
3	5.429	40	0.167	0.907
4	5.835	50	0.200	1.167
5	6.502	60	0.250	1.626
6	7.628	70	0.333	2.540
7	9.668	80	0.500	4.834
8	14.002	-	-	-

*Includes 500 µL HPLC sample removal

The resulting precipitated pellets were individually dried under vacuum at 40°C, cooled and redissolved in 1000 µL n-butanol using gentle vortexing. After allowing the samples to sit for 15 minutes at ambient temperature on the bench, the samples were centrifuged at 14 000 RPM for 5 minutes to allow for removal of the water and undissolved $(NH_4)_2SO_4$ salt as a precipitate at the bottom.

For HPLC analysis of lipopeptide quantities present in the precipitate of each salt fraction, 900 µL of the n-butanol solvent was removed after centrifugation without disturbing the $(NH_4)_2SO_4$ precipitate in the tube and again dried under vacuum at 40 °C. Finally, 900 µL of a 50% (v/v) acetonitrile:water was added, the sample filtered through a 0.22 µm syringe filter and used in RP-HPLC analysis.

Samples were also tested by Ion chromatography HPLC (IC) to determine the total $(NH_4)_2SO_4$ concentration for each fraction for temperature correction. During precipitation 500 µL of the supernatant was sampled after $(NH_4)_2SO_4$ and centrifugation, labelled and stored on ice. Divalent metal ions present in the culture media was removed with a concentrated 18 mM Na_2CO_3 IC buffer and the resulting sample diluted with distilled water to below 200 ppm SO_4^{2-}. The samples were filtered through 0.22 µm syringe filters before HPLC analysis. IC results as g/L SO_4^{2-} were used to calculate the total salt percentage in each fraction using the equation below where $\%(NH_4)_2SO_4$ is the percentage salt concentration achieved by addition of a saturated solution of 4.1 M $(NH_4)_2SO_4$ at 25°C.

Equation 3
$$\%(NH_4)_2SO_4. = \frac{[SO_4^{2-}]\,/96.06\;Mw_{SO_4^{2-}}}{4.1\;M\;(NH_4)_2SO_4,\;\;25°C} \times 100$$

3.9.3 Solvent Extraction

Growth media from *Bacillus amyloliquefaciens* cultures in late exponential stage, ±24 hrs after inoculation, were subjected to centrifugation to remove cells and the supernatant kept for solvent extraction. Before extraction was performed 20 mL of the supernatant was filtered with a 0.22 µm syringe filter to remove any residual bacteria and spores.

Solvent extraction samples were prepared for a “single-step” solvent extraction procedure, using different organic solvents (1-butanol, 2-butanol, isobutanol,

dichloromethane, diethyl ether, tert-butyl-methyl-ether (TBME) and n-hexane) at 50:50 ratios of solvent to sample (1ml solvent to 1ml cell free supernatant). Untreated cell free supernatant was kept as a reference sample for calculation of the lipopeptide baseline concentration present before solvent extraction.

The organic solvent-supernatant mixtures were mixed through gentle vortexing, transferred to a glass Pasteur pipette and allowed to settle and separate for 1 hour. The Pasteur pipette served as a separations funnel to separate the solvent phase from the water phase. The collected solvent phase samples were evaporated under normal atmospheric pressure using a jet of nitrogen gas, leaving behind the dried media compounds that were extracted.

After evaporation 2 mL of 50% (v/v) acetonitrile:water solution was added to the dried compounds in the organic solvent phase samples and 1mL 100% acetonitrile to the baseline concentration and water phase samples to give 2 mL samples of each sample for concentration comparison.

RP-HPLC was carried out to determine the amount of lipopeptides present in the baseline, water and solvent phase samples.

The distribution ratio of antifungal lipopeptides to surfactin and selectivity factors for each were calculated using the equations below:

Equation 4 $$Distribution\ Ratio\ (D_x) = \frac{[Organic\ Phase]}{[Water\ Phase]}$$

Equation 5 $$Separation\ Factor\ (\beta_x) = \frac{D_{Antifungals}}{D_{Surfactins}} = \frac{D_a}{D_s}$$

3.9.4 Thin Layer Chromatography (TLC) and Partial Purification of Antifungal Lipopeptides

Lipopeptide separations were performed on preparative silica TLC plates (20 x 20 cm, F254 silica, Merck, Germany) using a solvent system comprising of n-butanol, ethanol, water and acetic acid in the ratio 24:48:24:4. Crude lipopeptides isolated and concentrated through similar techniques used in n-butanol solvent extraction (section) were spotted onto TLC plates using a fine glass Pasteur pipette through

small additions and sequential drying of the plate at 40°C to concentrate the sample on the plate.

The plates were allowed separated at room temperature in an enclosed glass tank that was left to equilibrate for 30 minutes before the plates were added in the separation solvent described above.

After separation and 24 hours of drying under vacuum at 40°C, the plates were developed using water misting, UV_{380nm} and lastly spraying with a solution of 0.3% (w/v) of ninhydrin in 70% (v/v) ethanol. The ninhydrin treated plates were incubated at 80°C for 30 minutes to allow for spots to develop and then inspected for the presence of free amino acid groups.

For consistency, the retention factor (R_f) for each compound isolate displaying inhibition was calculated using Equation 2. This allowed for a universal technique to identify similar compound isolates, but separated on different TLC plates using the same solvent system regardless of separation time.

Compound displaying inhibition were obtained from untreated TLC plates by removing the silica corresponding to the R_f inhibition bands with a micro spatula and transferring the removed silica to a 4 mL HPLC vial. For extraction of the compounds contained in the silica matrix, the sample was washed in 1 mL of acetonitrile, centrifuged at 2000 RPM for 10 minutes, filtered through 0.22 µm syringe filters and analysed using RP-HPLC.

Chapter 4

RESULTS AND DISCUSSION

4.1 PHYTOPATHOGEN ISOLATION AND IDENTIFIATION

South African table grapes infected with various phytopathogens were supplied from farms in the regions of the Hex River Valley and from the Swartland by the South-African Table Grape Industry (SATGI).

A total of 79 crude filamentous isolates were collected from the post-harvest grapes supplied, including one isolate that displayed the properties of yeast, and sub-cultured in triplicate, through 5 rounds of sub-culturing on two different agar media types, to produce 59 pure cultures. Of these 59 pure cultures cultures, 16 out of 18 cultures submitted for DNA sequencing were successfully sequenced and the obtained sequences aligned using web software on the NCBI website, www.ncbi.nlm.nih.gov/.

To do DNA identification, 144 manual DNA alignment queries had to be performed and the resulting tables analysed individually to identify the genus. This was combined with 76 manual queries for inter-genus analysis using the same manual approach. Figure 4-1 demonstrates only the first few results from an alignment query which is only a fraction of the data obtained from one sequence alignment. This needed to be critically assessed in combination with morphology

results to identify the genus. Figure 4-2 shows the graphical alignment of one of the results in Figure 4-1 used to analyse a specific fragment in the alignment.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Fungal endophyte sp. q18 18S ribosomal RNA gene, partial sequer	887	887	93%	0.0	96%	HM537028.1
Uncultured soil fungus clone A17 18S ribosomal RNA gene, partial	887	887	93%	0.0	96%	HM037653.1
Uncultured soil fungus clone A12 18S ribosomal RNA gene, partial	887	887	93%	0.0	96%	HM037648.1
Uncultured ascomycete ITS region including 18S rRNA gene, ITS1,	887	887	93%	0.0	96%	AM901713.1
Uncultured fungus 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, I	887	887	93%	0.0	96%	AM260931.1
Sclerotinia sp. 5/97-18 ITS1, 5.8S rRNA gene and ITS2, isolate 5/97	887	887	93%	0.0	96%	AJ279480.1
Uncultured Botryotinia clone 2S-39 small subunit ribosomal RNA g	885	885	92%	0.0	96%	KF156272.1
Botryotinia fuckeliana isolate Bot_1283 18S ribosomal RNA gene, p	885	885	92%	0.0	96%	EF207415.1
Botryotinia fuckeliana isolate Bot_1285 18S ribosomal RNA gene, p	885	885	92%	0.0	96%	EF207414.1
Botryotinia fuckeliana isolate Bot_1284 18S ribosomal RNA gene, p	885	885	92%	0.0	96%	EF207413.1
Botryotinia fuckeliana strain t08016b1 18S ribosomal RNA gene, pa	883	883	92%	0.0	96%	JX875916.1
Botryotinia fuckeliana isolate RASG-6 18S ribosomal RNA gene, pa	883	883	92%	0.0	96%	JX840481.1
Botryotinia fuckeliana isolate RSL-1 18S ribosomal RNA gene, parti	883	883	92%	0.0	96%	JX840480.1
Botryotinia fuckeliana isolate BC12 18S ribosomal RNA gene, parti	883	883	93%	0.0	96%	GU724512.1
Botryotinia fuckeliana isolate Bf.ind09 18S ribosomal RNA gene, p	883	883	92%	0.0	96%	GU395993.1
Botryotinia fuckeliana isolate AFTOL-ID 59 internal transcribed spa	883	883	92%	0.0	96%	DQ491491.1
Botryotinia fuckeliana strain WA0000019044 18S ribosomal RNA g	881	881	93%	0.0	96%	JX981485.1
Uncultured fungus clone F3-O7 18S ribosomal RNA gene, partial sl	881	881	93%	0.0	96%	JX984719.1
Uncultured Sclerotiniaceae clone 6_d19 18S ribosomal RNA gene,	881	881	93%	0.0	96%	HQ211718.1
Uncultured ascomycete ITS region including 18S rRNA gene, ITS1,	881	881	93%	0.0	96%	AM901946.1
Uncultured fungus clone L042885-122-065-G02 internal transcribe	880	880	92%	0.0	96%	GU054208.1

Figure 4-1 Example of alignment results obtained from one DNA fragment sceened using DNA sequencing results on NCBI.

Isolates 3 and 6 could not be sequenced from DNA extracts using the ITS primers and subsequently they were identified using their macro- and micromorphology obtained from microscopic analysis.

Using these 144 DNA alignments the genera could be determined based on the highest alignment score returned from NCBI online. Alignment parameters used in each identification also yielded possible species to the genus if the full genetic sequence was available in the NCBI database, but care should be taken with such deductions as the nuclear ribosomal small subunit has poor species-level resolution in fungi (Schoch, et al., 2012).

The highest alignment scores linking to the genera were used in connection with micro and macro identification to identify the genera. The alignment score was automatically calculated by an algorithm in the software that used the number of identical nucleotides, mismatches and non-aligned nucleotides in each fragment of DNA to determine the fragment matches with the most similarities.

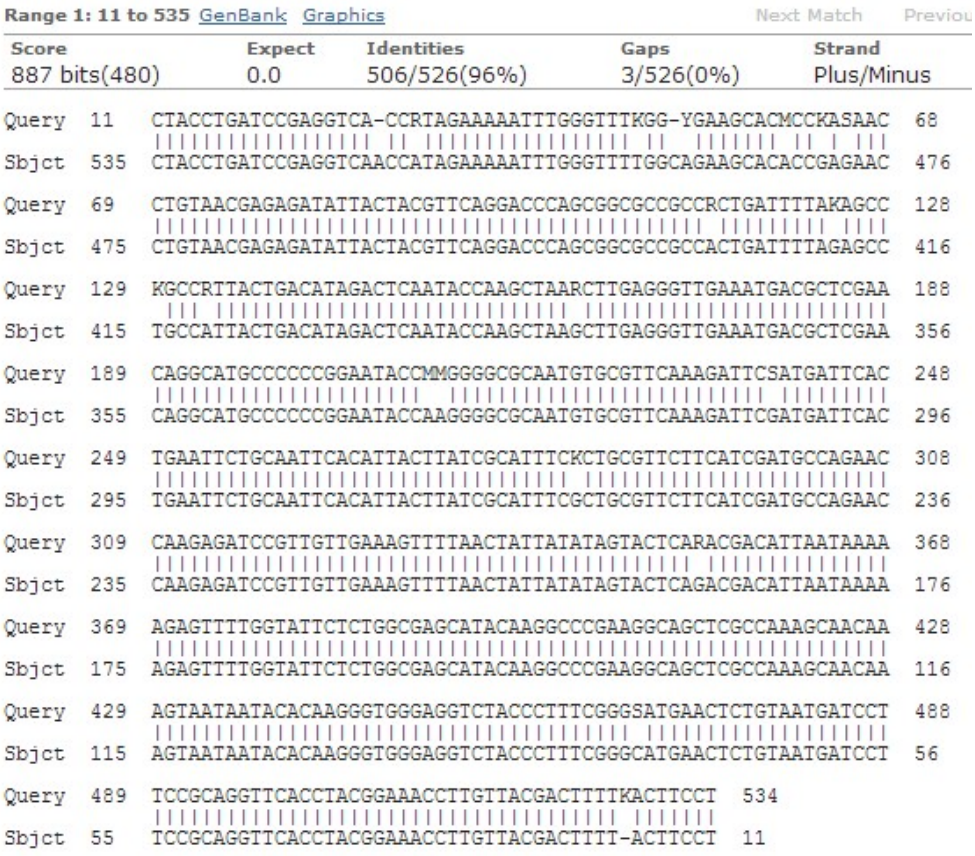

Figure 4-2 Graphical representation of alignment score from alignment results between sequenced fungi and NCBI's DNA sequence database.

Fungi exhibit 3 stages (morphs) of growth based on sexual or asexual reproduction observed. The specific morphs of the isolates are detailed in the genetic analysis (Table 4-1). For discussion all concluded data from genetic and morphology studies were grouped under their holomorphic name for simplicity. Thus the morphs *Botrytis*, *Botryotinia* and *Sclerotinia* spp. were classified their under their holomorph, *Botrytis*. Thus fungal pathogens are referred to in the holomorphic form in the text, Table 4-1, Table 4-5 and Figure 4-13.

Table 4-1 Genetic results of phytopathogens from DNA fragments via ITS 4 and ITS 5 PCR amplification

Isolate	Genus	Possible Species	PCR Fragment	Fragment Length	Fragment Covered (%)	Identity (%)	Alignment score	Differential Analysis
1	*Lewia*	*infectoria*	ITS 4	597	97	99	1059	N/A
	Lewia	*infectoria*	ITS 5	597	97	99	1070	N/A
2	*Botrytis*	*fabae*	ITS 4	538	97	99	948	>99
	Botryotinia	*fuckeliana*	ITS 5	540	97	99	961	>99
3*		-	ITS 4	-	-	-	-	N/A
	-	-	ITS 5	-	-	-	-	N/A
4	*Penicillium*	*expansum*	ITS 4	580	97	99	1037	>99
	Penicillium	*expansum*	ITS 5	581	98	99	1038	>99
5	*Alternaria*	-	ITS 4	570	98	99	1024	>99
	Alternaria	*brassicae*	ITS 5	571	98	99	1027	>99
6*		-	ITS 4	-	-	-	-	N/A
	-	-	ITS 5	-	-	-	-	N/A
7	*Botrytis*	*fabae*	ITS 4	533	97	99	953	>99
	Botrytis	*fabae*	ITS 5	536	98	99	959	>99
8	*Sclerotinia*	-	ITS 4	563	93	96	887	96
	Sclerotinia	*sclerotorium*	ITS 5	567	94	90	747	96
9	*Botrytis*	*fabae*	ITS 4	538	97	99	953	>99
	Botryotinia	*fuckeliana*	ITS 5	538	97	99	961	>99
10	*Botrytis*	*fabae*	ITS 4	538	98	99	955	>99
	Botryotinia	*fuckeliana*	ITS 5	542	97	99	972	>99
11	*Penicillium*	*expansum*	ITS 4	581	96	100	1038	>99
	Penicillium	*expansum*	ITS 5	583	94	100	1022	>99
12	*Penicillium*	*commune*	ITS 4	585	96	100	1048	>99
	Penicillium	*commune*	ITS 5	581	96	99	1018	>99
13	*Penicillium*	*commune*	ITS 4	580	97	99	1037	>99
	Penicillium	-	ITS 5	583	96	99	1031	>99
14	*Penicillium*	-	ITS 4	577	97	99	1022	>99
	Penicillium	*brevicompactum*	ITS 5	583	97	99	1031	>99
15	*Penicillium*	*expansum*	ITS 4	581	97	99	1029	>99
	Penicillium	*expansum*	ITS 5	582	95	100	1022	>99
16	*Penicillium*	*expansum*	ITS 4	582	97	99	1038	>99
	Penicillium	*expansum*	ITS 5	586	94	100	1016	>99
17	*Penicillium*	*expansum*	ITS 4	581	97	99	1040	>99
	Penicillium	*expansum*	ITS 5	581	98	99	1031	>99
18	*Penicillium*	*expansum*	ITS 4	579	96	99	1026	>99
	Penicillium	*expansum*	ITS 5	582	96	99	1022	>99

*The isolate could not be sequenced and identification was based on culture morphology only.

Wet slides prepared from actively growing plates of isolates 1-18 revealed that isolates 2, 3, and 7 -10 (Table 4-1) all had segmented hyphae and shared the same conidiophore shape with pear shaped spores. These cultures also had a fluffy brown texture that resembled wool. This is strong indication that these four cultures belonged to the *Botrytis* spp. Results from genetic sequencing (Table 4-1) gave fragment lengths between 538-563 base pairs for cultures 2, 7-10 with the total percentage of these fragment lengths covered during alignment with the database between 93% - 97%.

In addition to database alignments the results for *Botrytis* spp. isolates was confirmed by inter-genus DNA sequence alignments for isolates 2, 7-10 against each of the other isolates (Table 4-2) where isolates 2, 7, 9 and 10 had >99% identity through inter-genus differential analysis. However, isolate 8, *Sclerotinia* the telomorph of *Botrytis*, yielded only a 96% identity against cultures 2, 7, 9 and 10 (Table 4-2). Isolate 3 did not produce PCR products (Figure 4-3) and thus no DNA fragments were obtained for sequencing to include in analysis and no genus/specie could be derived as this was from DNA alignment results (Table 4-1).

Table 4-2 Differential analysis comparing the DNA fragments of cultures in the same genus, *Botrytis*.

***Botrytis* spp.**

ITS 4 DNA Fragment	Culture	2	7	8	9	10
	2	100	99	96	99	99
	7		100	96	99	99
	8			100	96	96
	9				100	99
	10					100

ITS 5 DNA Fragment	Culture	2	7	8	9	10
	2	100	99	90	99	99
	7		100	90	99	99
	8			100	90	90
	9				100	99
	10					100

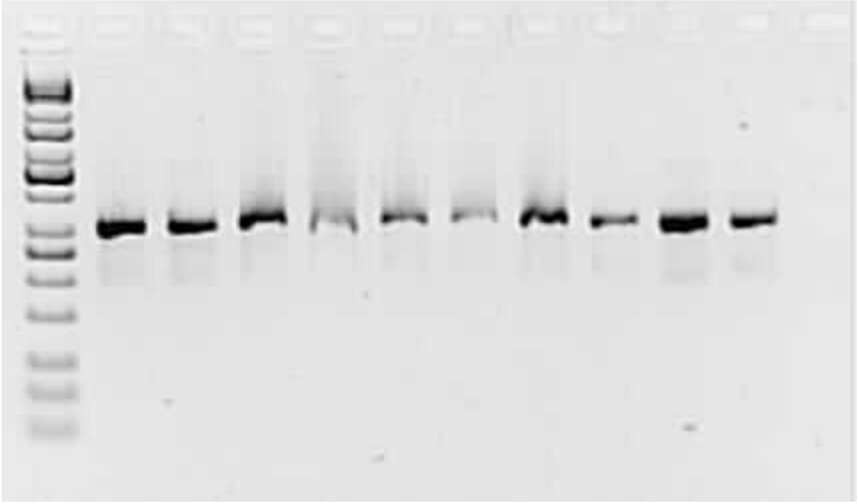

Figure 4-3 PCR products of successful ITS region amplification. From left to right: KAPA Universal Ladder depicting fragment length, Isolate 11, 12, 13, 14, 2, 4, 15, 16, 17, 18, Control. Isolates 3 and 6 could not be amplified (Image Provided by Leisl Brand, CAF Stellenbosch).

Isolates 4, 11-18 (Table 4-1) all had a green powdery appearance and microscopic slides of the isolates revealed conidiophores with brush-like strands with round spores characteristic of *Penicillium* spp. This genus was confirmed by DNA alignments (Table 4-1). Fragment lengths between 577-586 base pairs were achieved and was found to match between 99% - 100% of the NCBI database for *Penicillium* spp. with 93% - 97% of the fragment lengths aligned.

Similar to the inter-genus analysis of *Botrytis* spp. isolates (Table 4-2) differential inter-genus analysis of DNA fragments aligned against each other yielded >99% match for all *Penicillium* spp. isolates, results given in Table 4-3.

Isolate 6 also had conidiophores similar to those of *Penicillium* spp., but were not branched or brush-shaped. Instead it formed a vesicle surrounded by strands of conidiospores. This in combination with the black spot plate morphology indicated the *Aspergillus* spp. DNA could not be obtained for sequencing through the use of the standard DNA isolation protocol and thus no data was available for genetic studies or determination of a possible strain (Table 4-1). Figure 4-4 shows microscopic identification of isolate 3 and 6 compared to fungi references.

Table 4-3 Differential analysis comparing the DNA fragments of cultures in the same genus, *Penicillium*.

Penicilllium spp.

	Culture	4	11	15	16	17
ITS 4 DNA Fragment	4	100	100	99	99	99
	11		100	99	99	100
	15			100	99	99
	16				100	99
	17					100

	Culture	4	11	15	16	17
ITS 5 DNA Fragment	4	100	99	99	99	99
	11		100	99	99	100
	15			100	99	100
	16				100	99
	17					100

Table 4-4 Differential analysis comparing the DNA fragments of *Lewia* spp. and *Alternaria* spp.

Alternaria spp. vs. _Lewia_ spp.

	Culture	1	5
ITS 4 DNA Fragment	1	100	93
	5		100

	Culture	1	5
ITS 5 DNA Fragment	1	100	93
	5		100

Isolate 5 produced a fluffy white-grey mass macroscopically and microscopic analysis revealed light brown conidiophores displaying compartmented conidia. This is unique to the genus *Alternatia* and was confirmed with DNA alignments (Table 4-1) that yielded a 99% match to the NCBI database for other sequenced *Alternaria* spp.

Isolate 1 yielded two 597 base pair fragments and 97% coverage of DNA alignments yielded 99% identify to *Lewia infectoria*. Morphology studies of the plate yielded a white cotton-like texture with no visible spores. Microscope analysis could not identify any identifiable micro structures, but since both ITS fragment alignments yielded the same genus and specie with >99% identity *Lewia* spp. is a likely candidate. Table 4-4 shows the results of cross comparison of *Lewia* spp results with *Alternaria* spp. results displaying high comparison. This could be explained through Perelló & Sisterna (2008) documenting *Lewia infectoria* as the telomorph (displaying a sexual reproduction stage) of *Alternaria infectoria,* the anamorph (lacking a sexual reproduction stage).

In total, a good overview of the isolated fungi is given. Of the cultures sequenced, 50% were identified as *Penicillium* spp., 33% *Botrytis* spp. and *Alternaria*., *Aspergillus* and *Lewia* spp. combined as 17% other. Thus, isolation of antifungals from *Bacillus* spp. was the focus in this study.

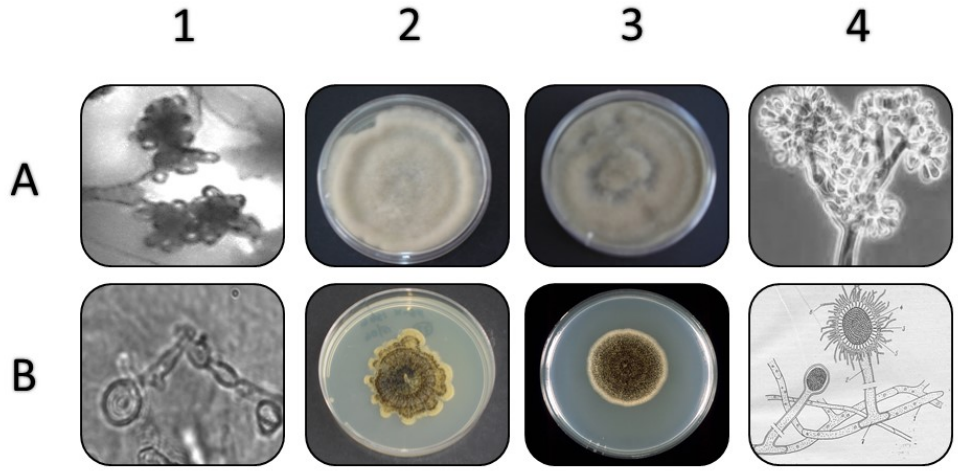

Figure 4-4 Culture morphology and microscopic analysis on A) isolate 3 and B) isolate 6 which indicates that Isolate 3, A1-2, resembles that of Isolate 10, A3, and reference slide A4. This indicates *Botrytis*. Isolate 6, B1-2, has high similarity with reference slides A3-4 of *Aspergillus niger*.

4.2 PHYTOPATHOGEN DISEASES

Phytopathogens identified through rigorous DNA alignments and morphology studies are linked to their relevant diseases in Table 4-5. All the fungal cultures are presented in their holomorpic form, i.e *Botryotinia* (anamorph of *Botrytis*, displaying asexual reproduction) and *Sclerotinia* (telomorph of *Botrytis* displaying sexual reproduction*)* are presented as their holomorphic designation, *Botrytis*.

Table 4-5 Identified phytopathogen isolates based on morphology and genetic sequence alignments and associated diseases. (Bautista-Baños, 2014)

Isolate	Geuns	Possible Species	Disease
1	*Lewia*	*infectoria*	Leaf Bight (Perelló & Sisterna, 2008)
2	*Botrytis*	*fabae*	Grey Mould/Noble Rot
3	*Botrytis*	-	Grey Mould/Noble Rot
4	*Penicillium*	*expansum*	Blue-Green Mould
5	*Alternaria*	*alternata/brassicae*	Bunch Rot (Swart, et al., 1995)
6	*Aspergillus*	-	Black Mould
7	*Botrytis*	*fabae*	Grey Mould/Noble Rot
8	*Botrytis*	-	Grey Mould/Noble Rot
9	*Botrytis*	*fabae*	Grey Mould/Noble Rot
10	*Botrytis*	*fabae*	Grey Mould/Noble Rot
11	*Penicillium*	*expansum*	Blue-Green Mould
12	*Penicillium*	*commune*	Blue-Green Mould
13	*Penicillium* spp.	*commune*	Blue-Green Mould
14	*Penicillium* spp.	*brevicompactum*	Blue-Green Mould
15	*Penicillium* spp.	*expansum*	Blue-Green Mould
16	*Penicillium* spp.	*expansum*	Blue-Green Mould
17	*Penicillium* spp.	*expansum*	Blue-Green Mould
18	*Penicillium* spp.	*expansum*	Blue-Green Mould

Botrytis spp., cultures 2,3,7-10, are largely responsible for gray /noble rot and is a significant problem among exporters of grapes, strawberries, apples and other fruit. *Penicillium* spp., isolates 4, 11-18, are pathogens that are predominantly the causative agents of blue/green mould. *Lewia* spp., isolate 1, and *Alternaria* spp., isolate 5, infects the leaves of plants and cause blight and black leaf spot reducing

growth and subsequent lower fruit delivery while *Aspergillus* spp., isolate 6, produce mycotoxins, aflatoxins and ochratoxin A, in foodstuffs that are carcinogenic to humans and animals, found especially in poorer countries.

4.3 SHAKE FLASKS EXPERIMENTS

4.3.1 Media Modifications

Lipopeptides produced by *Bacillus* spp. were to be investigated to determine the different lipopeptides expressed by four different stains. Research from Kim et al. (1997) documented a total lipopeptide yield of 4.5 g/L using a basic media composition as found in Table 4-6. Due to the high lipopeptide yield it reported, it was decided to use this media composition (labelled Kim media) as a baseline for further optimisation of media formulations towards lipopeptide production. The modifications are tabulated in Table 4-6 and Figure 4-5. Due to a lack of a standardised HPLC method for antifungal lipopeptide production at the time, a surfactin HPLC method using commercially available surfactin standards to gauge lipopeptide production was used in media development experiments Kim and A-F.

Table 4-6 Media compositions for trial experiments to monitor surfactin production of Bacillus subtilis ATCC 21332.

	Base	A	B	C	D	E	F	Reference
	Kim et al. 1997	Reactor	Shake Flask	Shake Flask	Shake Flask	Shake Flask	Shake Flask	Wei et al. 2007
Glucose	4% (w/v)	4% (w/v)	4% (w/v)	4% (w/v)	4% (w/v)	4% (w/v)	4% (w/v)	4% (w/v)
NH_4Cl	170 mM	170 mM	-	-	-	-	-	-
NH_4NO_3	-	-	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM
K_2HPO_4	60 mM	60 mM	1.26 mM	1.26 mM	6.1 mM	6.1 mM		-
KH_2PO_4	-	-	2.04 mM	2.04 mM	9.9 mM	9.9 mM	50 mM	50 mM
Na_2HPO_4	-	-	-	-	-	-	50 mM	-
NaH_2PO_4	12.5 mM	12.5 mM	-	-	-	-	-	-
$MgSO_4$	2 mM	2 mM	2.4 mM	2.4 mM	2.4 mM	2.4 mM	2.4 mM	2.4 mM
$MnSO_4$	0.3 mM	0.3 mM	0.01 mM	0.01 mM	0.01 mM	0.01 mM	0.01 mM	0.01 mM
$FeSO_4$	-	-	0.008 mM	0.008 mM	0.008 mM	0.008 mM	0.008 mM	0.008 mM
$CaCl_2$	-	-	7 µM	7 µM	7 µM	7 µM	7 µM	7 µM
Yeast Extract (w/v)	0.05%	0.05%	0.05 %	-	0.05 %	-	0.05 %	-

The modification to the Kim base media are presented in Table 4-6 using *Bacillus subtilis* ATCC 21332. Shake flask experiments with the Kim media yielded a total yield of 5.1 g/L surfactin. This was expected from reported results in literature, but

the product was found almost entirely as a precipitate with no product in the supernatant due to the significant drop in pH (<pH 3). This would make sampling of reproducible quantities difficult during shake flask experiments and it was decided to attempt modifications to allow the product to exist in the supernatant for easier isolation and quantitation. This resulted in 9 additional media formulations that were investigated towards this goal (Figure 4-5).

Using the same Kim media in a fully automated bioreactor at pH 7, designated Media A, to investigate the effect of pH control, caused the surfactin to stay in the liquid phase, but yielded a total of 0.108 g/L of surfactin with a CDW of 2.21g/L.

The reason for the discrepancy between yield in shake flask versus bioreactor regarding surfactin concentrations remains unclear, but could most likely be attributed to the lack of environmental stress on the *Bacillus* spp. to produce secondary products due to controlled pH and better aeration in reactor with ample nutrients available.

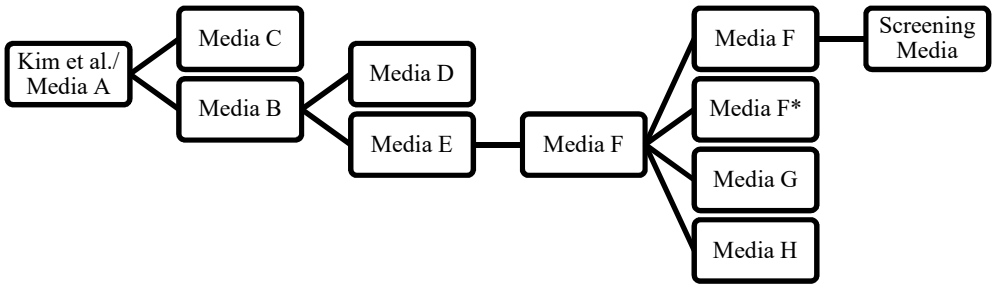

Figure 4-5 Flow chart indicating the process flow of steps taken for the optimisation of culture media to ensure lipopeptides are fully contained in the supernatant combined with high yield. The end result was used in screening experiments.

The results for the different media compositions will now be discussed, starting with Figure 4-6. The Kim media was modified to produce media B and C. This was done through substituting NH_4Cl for NH_4NO_3 to still provide ammonium with an added inorganic source of nitrate found to stimulate higher yields of lipopeptide production. Addition of calcium and iron was also made. A 3.3 mM phosphate

buffer at pH 7.2 was included with the addition of a KH_2PO_4 for maximum buffer capacity. Media C lacked the addition of yeast extract to investigate the need for an organic source of nitrogen for lipopeptide production. This modification further increased supernatant concentrations to 0.218 g/L in media B and 0.02 g/L in media C. It was concluded that the phosphate buffer was still too weak to prohibit pH precipitation and that organic nitrogen was important. Thus yeast extract as nitrogen source could increase lipoppepide production and the buffer concentration had to be increased to keep the pH from falling and causing precipitation.

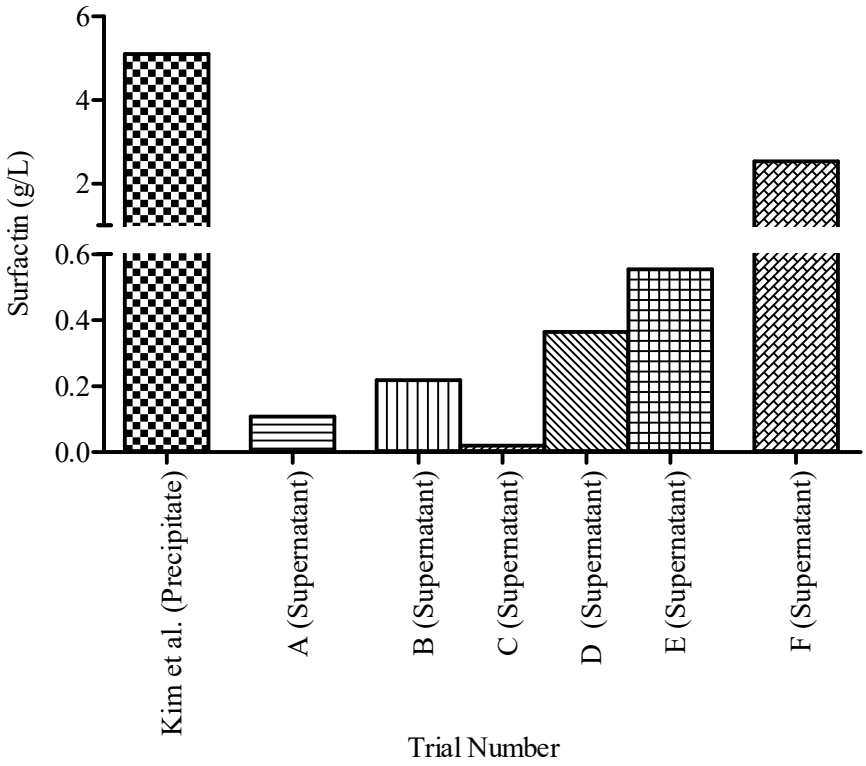

Figure 4-6 Media Trial Experiments, Kim and A through F detailing surfactin production of *B. subtilis*.

Media B and C was further modified to deliver media D and E, Figure 4-5, which had an increased phosphate buffer concentration of 16 mM which further increased the production to 0.364 g/L and 0.554 g/L respectively. The buffer capacity was found to be still too low to prevent pH precipitation from occurring. The increase in potassium also resulted in a negative effect on cell growth.

Media F was derived from D that included organic nitrogen from yeast extract, 0.05% (w/v), with the substitution of K_2HPO_4 with Na_2HPO_4 to decrease the amount of potassium in the media and increase the total buffer concentration to 100 mM. This change yielded 2.534 g/L surfactin in the supernatant and thus this media was chosen for further investigation of lipopeptide production of different strains of *Bacillus* spp.

It was also aimed to determine the location of lipopeptides in sample fractions, data which was missing from literature. Different fractions of media A samples, which yielded low supernatant results (Figure 4-6), were tested for lipopeptide isolation. To do this, *B. amyloliquefaciens* was cultured and fractioned into different sample constitutes and analysed with HPLC to determine the ratios of lipopeptides, Figure 4-7. This is the first known study to follow this approach and record data for the fractions.

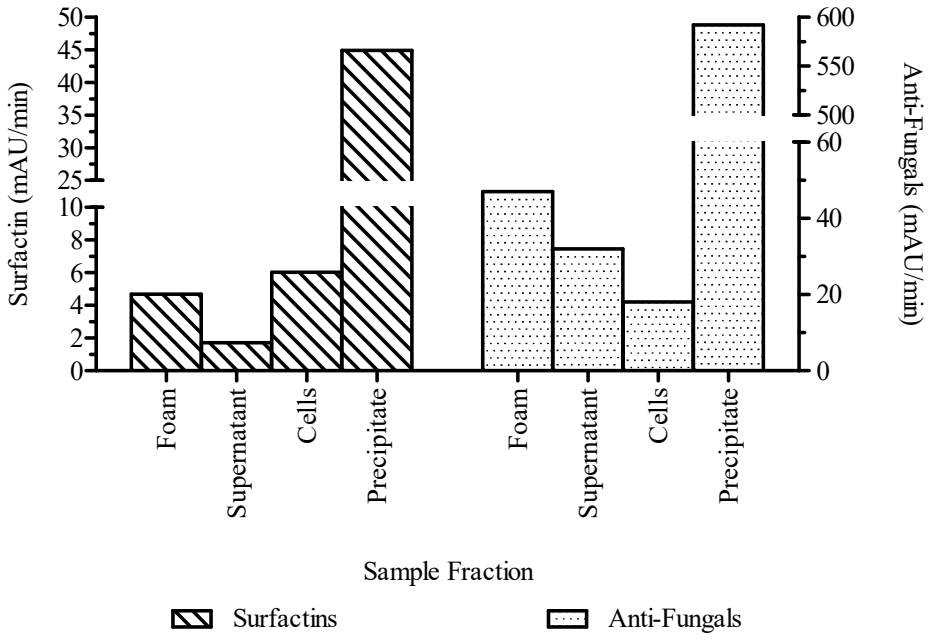

Figure 4-7 *B. amyloliquefaciens* lipopeptide distribution of media samples. Isolated culture samples were fractioned into their constitutes and analysed to compare the concentration in each fraction corresponding to antifungals and surcactins.

Calculating the lipopeptides present as a percentage of the total concentration in each family, it was found that antifungals were found in precipitate (85.92%), foam

(6.82%), supernatant (4.65%) and cells (2.62%) while surfactin was found in precipitate (78.30%), foam (8.18%), cells (10.51%) and supernatant (3.01%). Notably the cell fraction for surfactin contained four times the lipopeptide concentration in comparison to antifungals.

As was discussed, the lack of pH control allows the lipopeptides to precipitate out of the media and, if not tested, could yield erroneous results if only the supernatant fraction was tested to determine media efficiency, i.e. if supernatant from Figure 4-7 alone was tested to determine efficiency. This leads to ask the question how many papers might have reported "failed experiments", but in fact lost their product together with the cells during centrifugation. The most lipopeptides for both surfactin and antifungals was found in the precipitate. The cells also displayed potential to be isolated and extracted to allow a yield increase from fermentations and is comparable to the yield obtained from foam for processes interested in surfactin isolation.

In this study however, the media was developed with the goal to deliver all the lipopeptides of the foam and precipitate fractions combined into the supernatant to ease sampling, hence the reporting of supernatant values in Figure 4-6 and subsequent use of supernatant in radial diffusion assays.

4.3.2 Nitrate Addition

The results for nitrate addition are presented in Figure 4-8. Base media (F) yielded 1.3 g/L surfactin at a total nitrate concentration of 50 mM inorganic and 0.05% (w/v) yeast extract. Removal of the yeast extract produced media F* and severely affected lipopeptide production with a total concentration of 0.23 g/L surfactin.

Addition of 50 mM inorganic nitrate to media F to a final concentration of 100 mM produced media G and did not improve lipopeptide production and only yielded 0.46 g/L surfactin. Removal of the yeast extract in media G yielded media H with only inorganic nitrate at 100 mM and further degraded the lipopeptide production down to 0.14 g/L surfactin.

It can therefore be concluded that the addition of nitrate has an effect on lipopeptide production and that organic nitrogen sources in the form of yeast extract appears to be preferred above inorganic nitrate for surfactin production.

High concentrations of inorganic nitrate from its ammonia salt proved to have a negative effect on production in shake flasks possibly due to chemical toxicity.

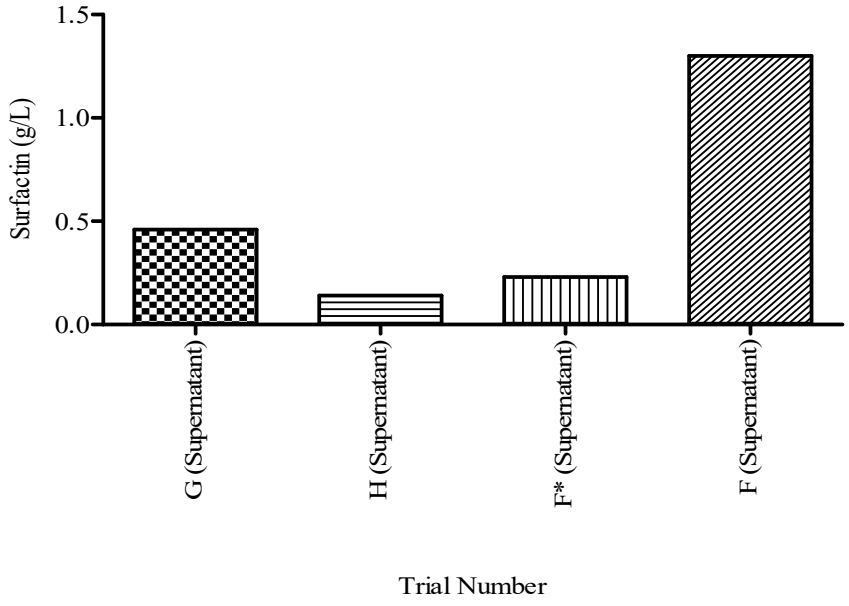

Figure 4-8. Surfactin lipopeptide results from screening experiments to investigate the addition of nitrate (NH_4NO_3) to base media. Media G (+NO_3,+Yeast Extract), media H (+NO_3,-Yeast Extract), Controls: Media E (-NO_3,-Yeast Extract), media F (-NO_3,+Yeast Extract).

4.3.3 *Bacillus* spp. Cultivation

Four strains of *Bacillus* spp. were cultivated in triplicate shake flasks for 7 days to investigate lipopeptide production and to isolate samples for efficacy studies detailed in section 4.4.

Media F was chosen based on results from media composition trials, Figure 4-6, and was used in cultivation experiments. The results for antifungal lipopeptide production are presented in Figure 4-9. Antifungal lipopeptides are quantified as arbitrary units (AU) for comparison due to the lack of a calibration curve for most lipopeptides where 1 AU = 1 mAU*min, the HPLC unit for peak results.

Bacillus amyloliquefaciens was found to be the best growing and highest antifungal producing isolate, Area 169 (mAU*min), of the four strains tested for the specific media composition with 47 lipopeptide homologs recorded. This data was

supported by similar research conducted with the same media composition in controlled reactor studies (Pretorius, 2014). The high production of antifungal lipopeptides is also attributed to the genetic advantage *B. amyloliquefaciens* have against the other strains, discussed in Chapter 2.

The low production of *B. subtilis* and *B. spizizzini*, at 0 mAU*min and 11 mAU*min respectively, was expected due to the genetic preference of these strains towards surfactin production. Other strains like *B. licheniformis* also fared poorly on the media composition and thus future efforts need to be directed towards media optimisation for these strains to produce their unique arsenal of antifungals. The results for *B. subtilis*, *B. spizizzini* and *B. licheniformis* is supported by similar findings reported by Pretorius, D (2014) using reactor studies and indicates the need to review the media composition for these strains.

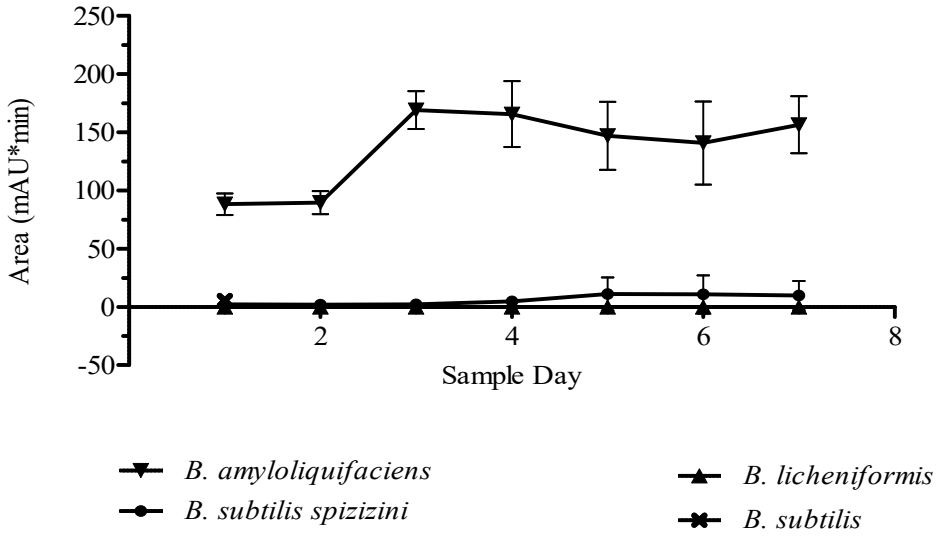

Figure 4-9 Total antifungal lipopeptide production of four *Bacillus* spp. strains from shake flask culturing (n=3).

To elucidate on the spectrum of different homologs produced, which lacks from literature, the production of each antifungal lipopeptide homolog over seven days of culturing for *B.amyloliquefaciens* is given in Figure 4-10.

The individual homologs relating to antifungal lipopeptides were labelled 1 – 41 (Figure 4-11) and using these labels in conjunction with the data in Figure 4-10, peaks 13, 16, 20, 21 and 22 were identified as the highest sustained during stationary phase, increasing in concentration from 0h and reaching a maximum after 24h of culturing and sustained throughout till day seven of sampling. This "fast" production could prove a valuable asset of *B. amyloliquefaciens* during large scale production.

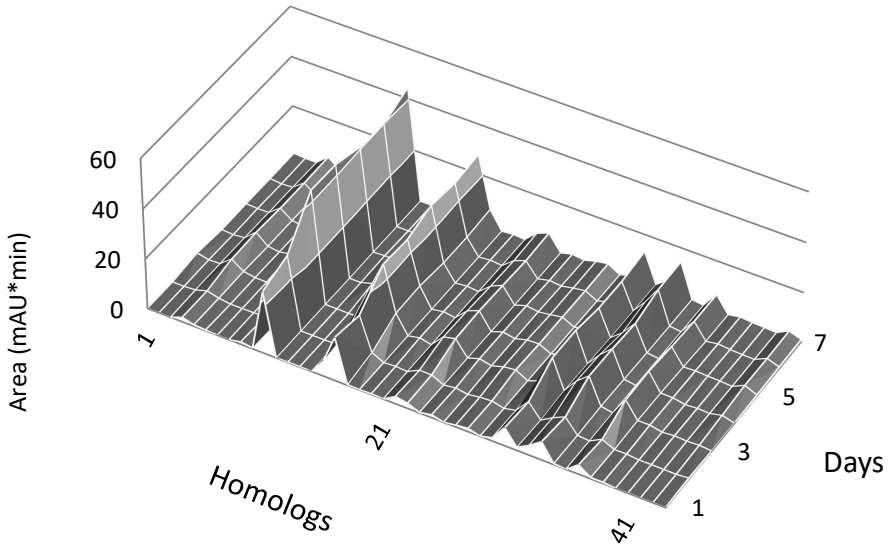

Figure 4-10 Average lipopeptide production from triplicate shake flask experiments of *B. amyloliquefaciens* showing the different homologs produced.

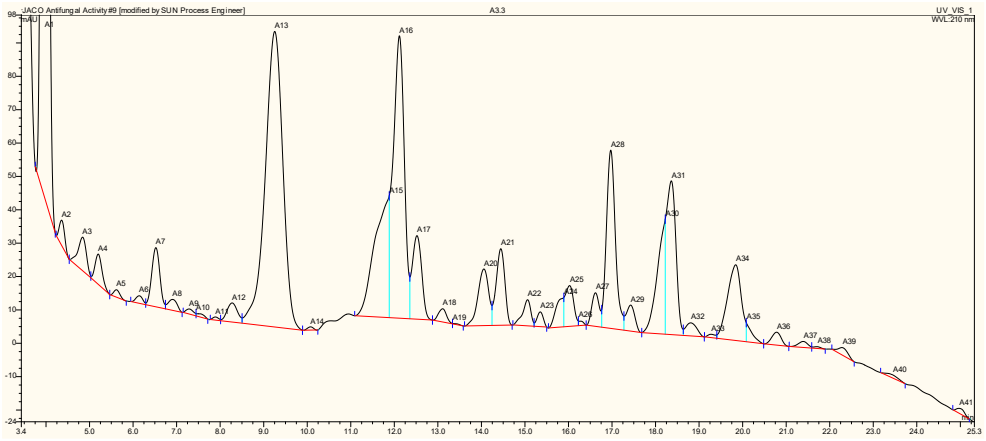

Figure 4-11 Chromatogram from *B. amyloliquefaciens* antifungal lipopeptides.

Iturin standards were analysed with HPLC and using the data each of the peaks from Figure 4-11 were correlated to the peaks of the Iturin standards. It was determined that antifungal lipopeptide peaks from *B. amyloliquefaciens* labelled 8, 12, 13, 16,17, 18, 21 and 22 showed similarities to Iturin peaks 1 through 9 respectively and is shown in Figure 4-12.

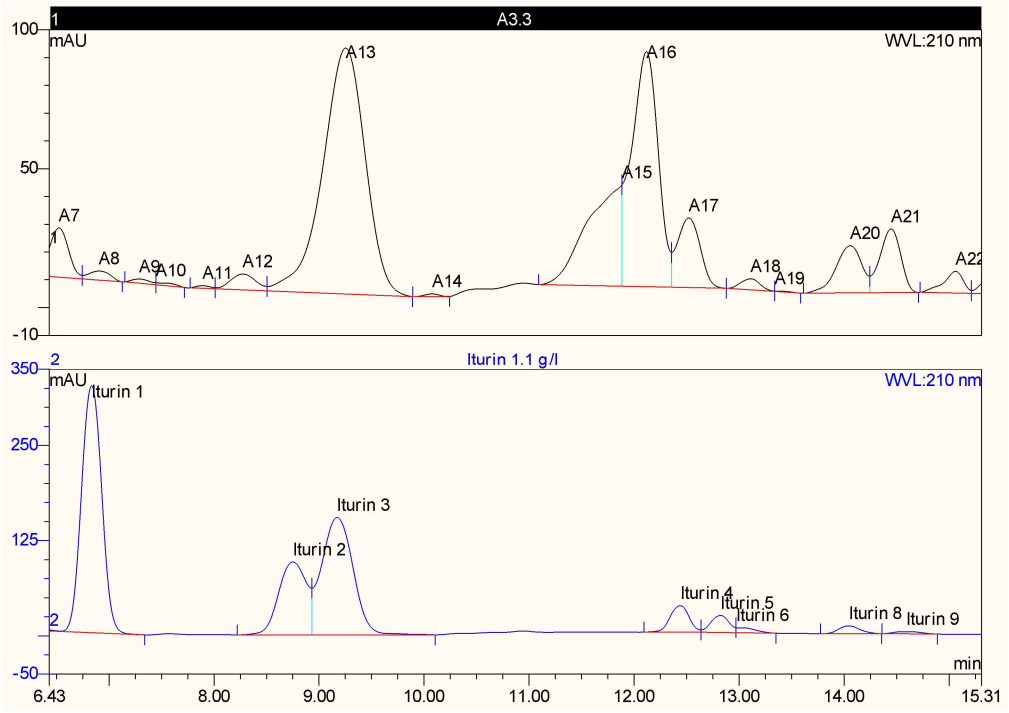

Figure 4-12 Chromatogram from *B. amyloliquefaciens* correlating peak name to peaks of Iturin standards.

This valuble data will be used in the next section, which will determine the efficacy of *B. amyloliquefaciens* lipopeptides on phytopathogens isolated from grapes, towards the goal of identifying antifungal activity and which antifungal lipopeptides are involved in antifungal activity.

4.4 BIOLOGICAL ACTIVITY STUDIES OF LIPOPEPTIDES

Crude supernatants containing lipopeptides were obtained from each of the four *Bacillus* spp. cultures during 7 days of cultivation on media F in triplicate (as detailed in section 4.3.3). Supernatants were sampled at specific time intervals from the triplicate shake flask experiments and tested for efficacy on 9 phytopathogens using radial diffusion assays as detailed in Section 3.8.1. This resulted in 756 wells, from more than 189 prepared agar well plates, which needed to be inspected, inhibition zones manually measured and inhibition zone diameter recorded.

Due to the size of the experiment, radial diffusion assays were the method of choice as the first preliminary screening approach to investigate the cocktail of lipopeptides produced in bulk and quantify the antimicrobial activity

.

4.4.1 Lipopeptide Anti-Microbial Activity

The supernatants of each of the four *Bacillus* spp. were tested for anti-fungal activity against 9 target organisms. The target organisms included the first 8 isolates identified and linked to their respective diseases and as well as *P. digitatum* DSM 22751. These were chosen on grounds of their different macro and micro morphology to attempt to cover a wide range of diseases.

Supernatant from *B. amyloliquefaciens* exhibited anti-fungal activity against all 8 of the isolates tested, as well as against *P. digitatum* from day 1 (Figure 4-13) while *B. subtilis spizizini* exhibited minor activity against one of the fungal isolates tested to date (isolate 8, *Botrytis* spp.) on days 5 to 7 (data not shown).The low antifungal activity of *B. subtilis spizizini was* due to corresponding low antifungal production, results which are shown in Figure 4-9.

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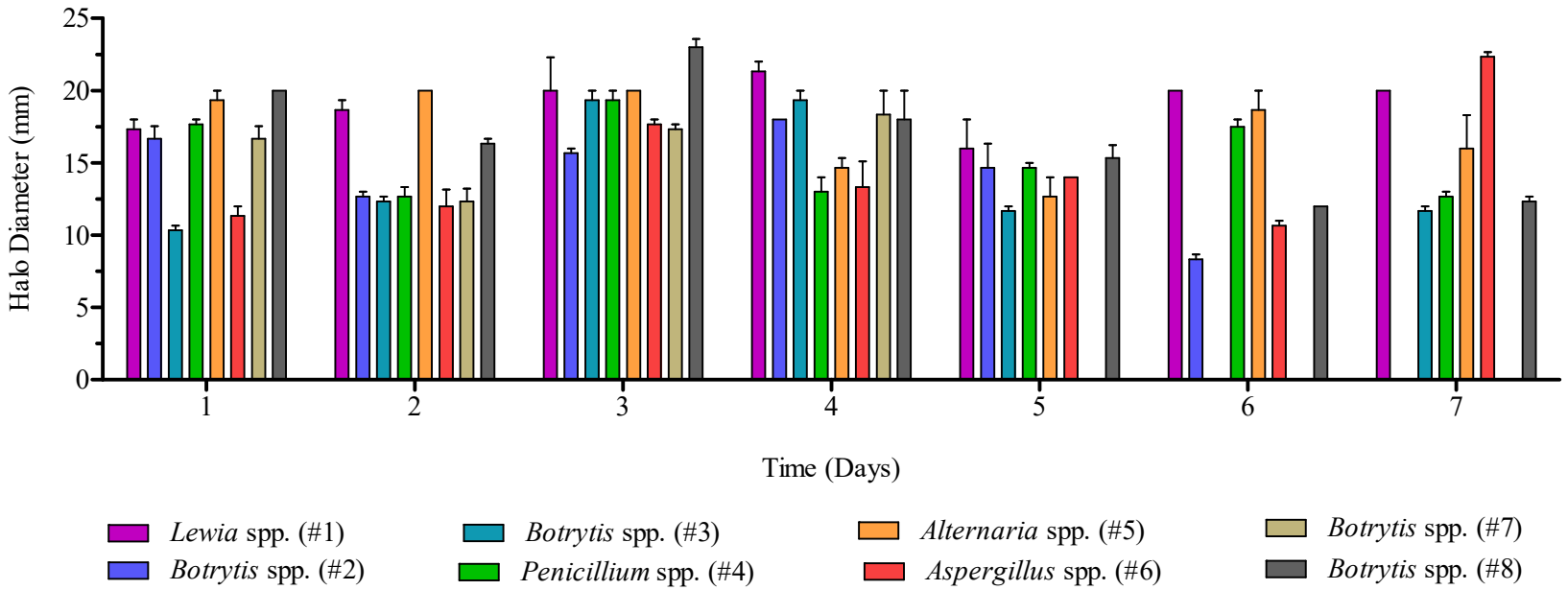

Figure 4-13 *B. amyloliquefaciens* antifungal activity on filamentous fungal isolates using crude supernatant from shake flask culturing experiments in triplicate over seven days. (Raw measurement data can be found in Addendum C)

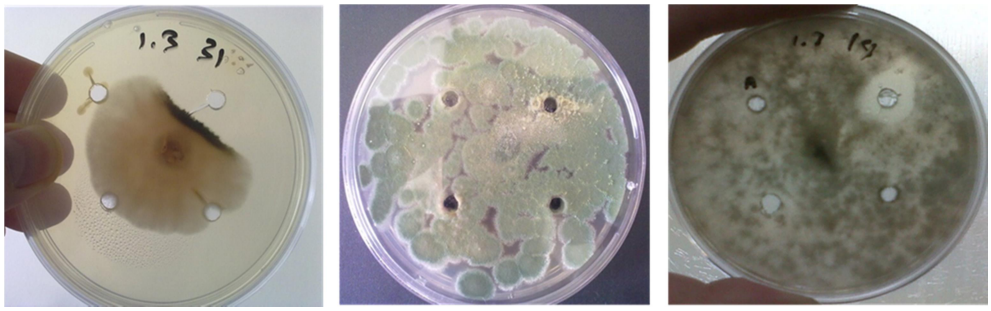

Figure 4-14 Antifungal activity of *Bacillus* lipopeptides on phytopathogenic fungi. From left to right: 1) Inhibition of *B.amyloliquefaciens* on *Alternaria spp*. #5 2) *Penicillium* spp. #4 showed no inhibition for *B. spizizini* and 3) Radial diffusion assay results results for *Botrytis* spp. #2 from,efficacy tests shows a clear halo due to the presence of antifungal lipopeptides produced by *B.amyloliquefaciens*.

The trends for all 8 phytopathogen isiolates can be seen maintaining activity from days 1 to 4. These results correspond to the increase seen in antifungal production in

Figure 4-9. With respect to the non-sporulating filamentous isolates tested in triplicate, the highest anti-fungal activity against the isolates was observed in isolate 8 on day 3 with a 24 mm halo, followed by isolate 6 on day 7 with a 23 mm halo (Figure 4-13). In isolate 1, anti-fungal activity (18-20 mm halo) stayed relatively constant throughout. Isolate 2 showed antifungal activity, reaching a maximum at day 4 with a 18 mm clear zone, but decreased towards day 7 when no halo was observed. Anti-fungal activity similarly decreased in isolate 7 from a 20 mm halo on day 4, disappearing completely on day 5. Oddly, *Botrytis* isolate 3 regained a low activity on day 7 of 11-12 mm inhibition halo.

The scatter of the anti-fungal activities would support the argument that individual homologues from a lipopeptide family are responsible for activity against individual fungal isolates. It also suggests from the results that a good day to draw samples for MIC studies would be on days 1 – 3 as these included activity against all the fungi isolates, likely corresponding to higher anti-fungal concentrations.

Thus future studies will be aimed towards reassessing anti-fungal activity based on individual homologs of each family of anti-fungal lipopeptides. This, combined

with a quantitative anti-fungal screening methodology (such as the minimum inhibition concentration assay) will more rigorously define the impact of these lipopeptides on specific phytopathogens.

The highest anti-fungal activity towards *P. digitatum* was exhibited on day 4 when a 12mm halo was observed (Figure 4-16). However, in contrast to the isolates, the halo around the well of the sporulating *P. digitatum* was not completely clear.

Microscope slides were prepared from the area outside and inside the halo areas (Figure 4-15). Normal vegetative growth was observed outside the halo (shown left) with conidiophores and mycelia growing normally. Within the inhibition halo, spores were visible but growth was stunted just after the formation of a germ tube (shown right). To test if these spores were still viable, a plug from the spores from within the halo were placed on new PDA agar and incubated for 3 days. Normal growth appeared and this indicated static inhibition of *P. digitatum.*

This static inhibition was in contrast to that of fungicidal activity that was observed at the concentrations tested in *Lewia* spp., *Botrytis* spp., *Penicillium* spp., *Alternaria* spp. and *Aspergillus* spp, results reported in Figure 4-13, and could

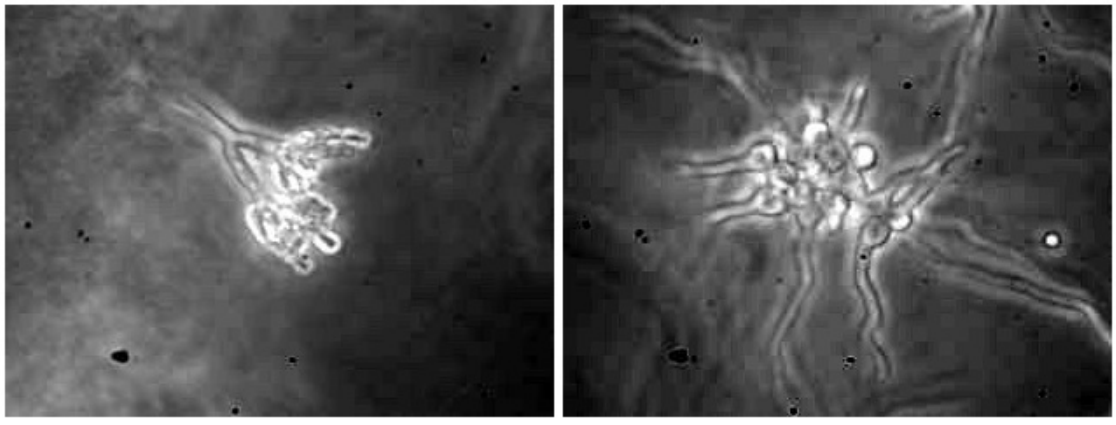

Figure 4-15 Microscopic imaging of *P. digitatum* DSM 2751 from PDA plates. Normal growth (left) with the formation of conidiophores were observed outside the halo, while mycelia growth was stunted just after the formation of a germ tube (right) following inhibition from *B. amyloliquefaciens* supernatant.

be an indication that static inhibition of *P. digitatum* could be as a result of too low antifungal lipopeptides present for this strain. This theory will have to be reviewed using higher dose dependent studies to investigate antifungal dose responses.

The static inhibition of *P. digitatum* could be a result of too low antifungal lipopeptides present for this strain and will have to be reviewed using higher dose dependent studies to investigate this theory.

It is interesting to note that looking at the data from inhibition and lipopeptide production, the trend of anti-fungal activity for *Penicillium digitatum* corresponds with that of the varying anti-fungal lipopeptide concentrations of *B. amyloliquefaciens* (Figure 4-16), suggesting that there is a concentration dependent effect.

However, from the data on day 5 to 7, the lipopeptide production does not completely correlate with activity and therefore it was concluded that the activity must be due to a varying concentration of certain homologs rather than total lipopeptide concentration. This was further investigated in section 4.7.

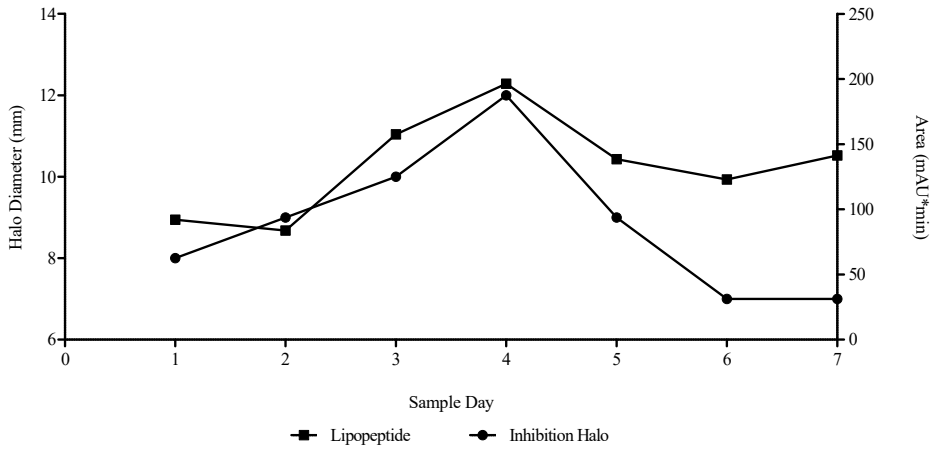

Figure 4-16 . *B. amyloliquefaciens* anti-fungal lipopeptide production *vs*. anti-fungal activity of *P. digitatum*

4.4.2 Haemolytic Activity

Radial diffusion assays using human blood agar were used to assess haemolytic activity of surfactin. *B. amyloliquefaciens* consistently displayed the highest values

of haemolysis throughout the duration of the experiment in the order of 8-10 mm (Figure 4-17, Figure 4-18). Haemolytic halos were observed on *B. subtilis* plates from day 3 and remained relatively constant in diameter, 6-8 mm, until day 7. A comparable curve was observed with *B. subtilis spizizini* from day 4 to 7 at a lower haemolytic activity of 2 mm halos. *B. licheniformis* displayed activity that increased rapidly from day 6 to seven, although the activity was not beta haemolysis (true hemolysis) but rather alpha haemolysis (partial haemolysis) from the green tint of the halo, usually caused by oxidisation from products such as H_2O_2.

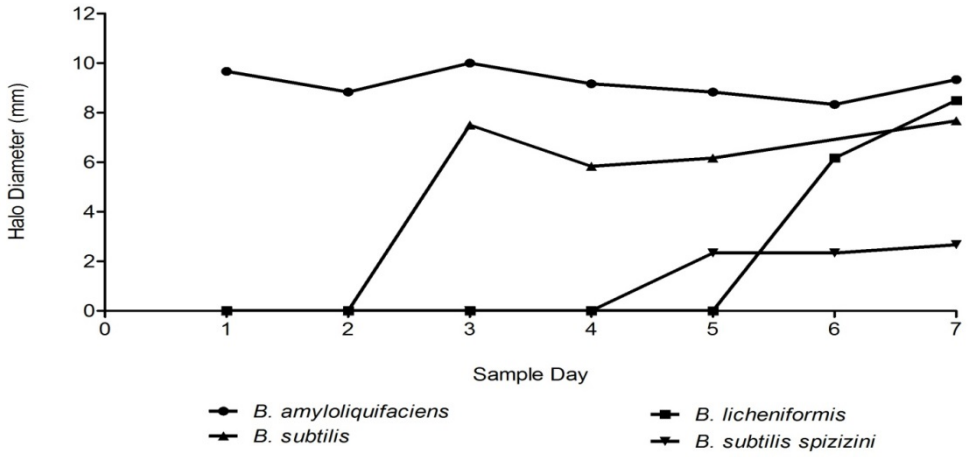

Figure 4-17. Inhibition halos of haemolytic activity recorded four the four *Bacillus* strains tested during shake flask culturing over seven days.

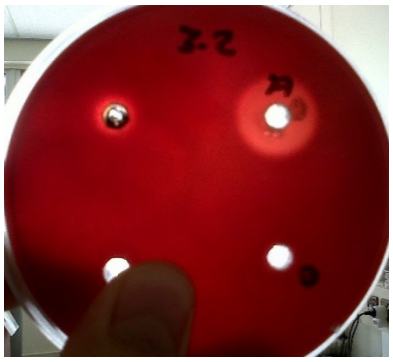

Figure 4-18 Blood agar plate showing haemolytic activity from surfactin production in *Bacillus amyloliquefaciens*, top left and right, and control (water) bottom right.

Ideally the final product should not include surfactin for application on edible food post-harvest, thus downstream processes should focus not only on the total isolation efficiency of the downstream methods for lipopeptide yields as a whole, but also the capability of the method to isolate the antifungal lipopeptides separately from surfactin. Downstream isolaton and purification results, relating to the separation efficiency of antifungals from surfactin, are further discussed later in this chapter.

4.4.3 Statistical Evaluation of Efficacy Data

Using the data provided through HPLC for lipopeptide production during cultivation (section 4.3.3, Figure 4-10) and efficacy halo diameters (section 4.4.1, Figure 4-13), the mathematical correlation between lipopeptide production and antifungal efficacy was evaluated.

Correlations between the lipopeptide production of each individual homolog and total lipopeptide production was evaluated against the inhibition trends of all phytopathogens tested to determine if a homolog or group of lipopeptide homologs are responsible for the observed activity.

It was found that most of the activity could be attributed to a combination of homologs and to a high extent some individual homologs (Table 4-7). Such was the case for instance with *Botrytis* spp isolates 7 and 8 with >92% correlation of being caused by a single lipopeptide. Other strains such as *Aspergillus* showed higher activity attributed to the combination of multiple homologs, 95.2%, than the possibility of the activity being due to a single homolog, 64%.

Table 4-7. The correlation between homologs being responsible for activity, either alone or in combination, towards phytopathogen inhibition.

Isolate	Individual Homolog	Combination
Lewia spp.	52.9%	73.5%
Botrytis spp.	75.2%	98.0%
Botrytis spp.	76.7%	91.1%
Penicillium spp.	66.1%	87.3%
Alternaria spp.	63.2%	74.6%
Aspergillus spp.	64.0%	95.2%
Botrytis spp.	95.6%	84.0%
Botrytis spp.	92.3%	91.8%

In Figure 4-19 below correlation results of antifungal homologs A1-A41 towards efficacy is presented, red indicating high correlation of production trends with compared efficacy measurements and blue having no to low contributions to activity.

In this graph it is clear that homologs in the area A3 through A21 are most abundant between all the fungal isolates with A13 being very prominent in almost all fungal isolates. The area of high activity links to the same area identified as iturins (Figure 4-12).

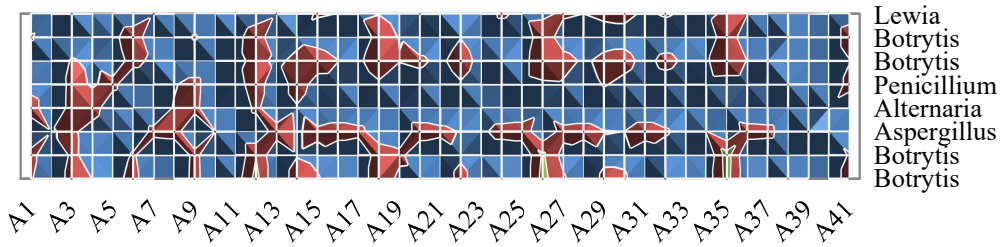

Figure 4-19. Spectrum of antifungal activity of antifungal homologs isolated from *B. amyloliquefaciens* A1 through A41. Red indicates high correlation between lipopeptide production trends versus antifungal activity curves and yellow to blue indicating low to no activity.

The statistical data presented in this section will be used and further discussed later in this chapter when links are drawn between homologs and observed antifungal activity from TLC plate isolations.

4.6 LIPOPEPTIDES ISOLATION AND CRUDE PURIFICATION

4.6.1 pH Precipitation

The first attempt to separate the complex composition of culture media into individual compounds and isolate purified lipopeptides was done through pH precipitation of media proteins which include lipopeptides. This was achieved by decreasing the pH of 30 mL fractions of cell free supernatant, obtained from an actively growing culture of *Bacillus* spp. in late exponential phase, in increments of 1 pH unit.

Titrations generally took around 30 minutes to complete and the total time the samples were allowed to precipitate at room temperature was 1 hour after the completion of titration to allow sufficient time for the precipitation step to take place.

During titration from high to low pH, with precipitate being removed after each pH increment of 1, looking at visual precipitation the formation of precipitate was first visually observed from <pH 5 with the highest amount of precipitate forming at ± pH 4 (Figure 4-20). From pH 4 to pH 1 the visual amount and rate of precipitation vastly decreased, with pellets being barely visible in fractions pH3 and below.

Contradictory to visual observations, HPLC data revealed an increase of lipopeptides isolated from precipitates below pH 3 and all the lipopeptides being removed in the fractions lower than pH 2. Fraction pH 2, with almost no visible pellet, had comparable amounts of lipopeptides to that of fraction pH 4 and thus it is concluded that, with a significant amount of precipitate visualised at pH 4 and almost no pellet in pH 2, fraction pH 4 contained more contamination from other proteins.

This would suggest that the purest amount of lipopeptides per mass precipitate was found in fraction pH 2 (vs. fraction pH 4) and had minimal contamination of the other proteins present in the media that co-precipitated in fraction pH 4. Due to the limitations of the HPLC method however, proteins other than lipopeptides in the fractions could not be identified and quantified and was beyond the scope of this study.

The total recovery of lipopeptides as a precipitate from all fractions was also calculated to be at 9.5% ± 1.0 for antifungal lipopeptides and 19.0% ± 7.9 for surfactin., which is much lower than the documented 55%.

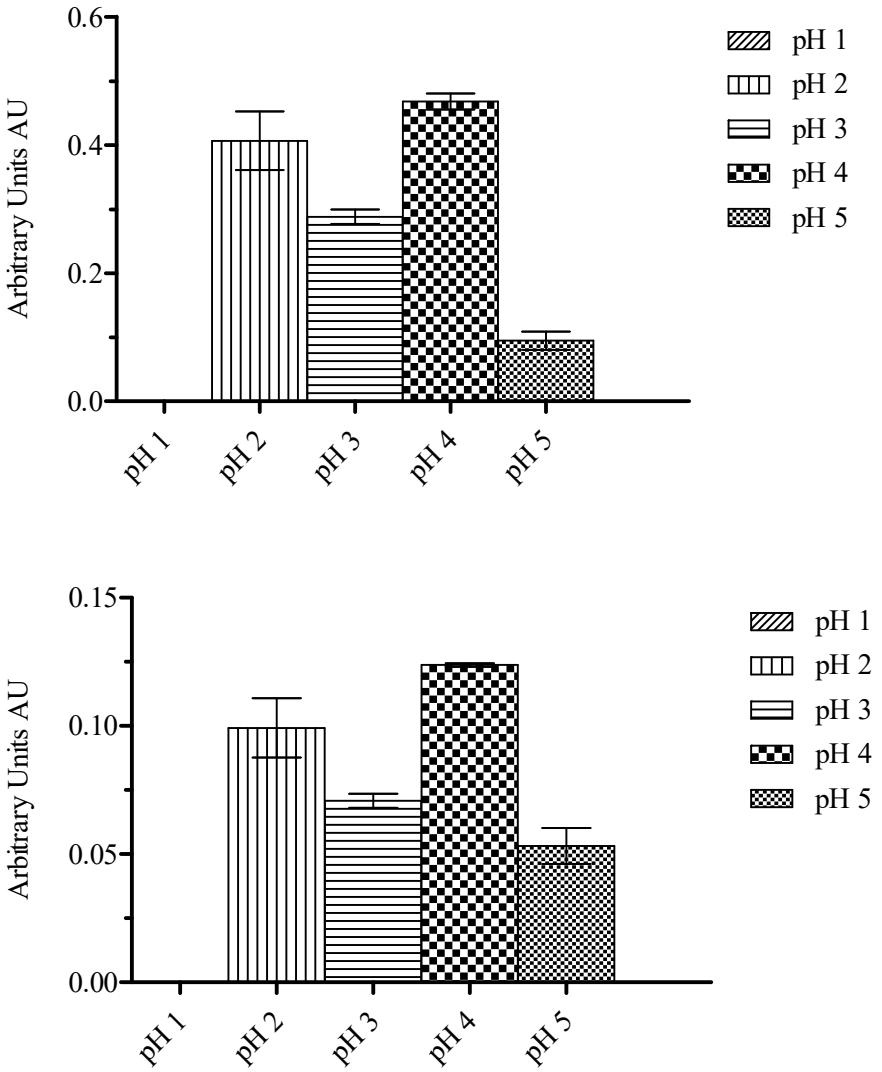

Figure 4-20 The investigation of pH precipitation of antifungal lipopeptides (top) and surfactin (bottom) to elucidate on process strategies for antifungal isolation and purification (n=2).

Adding acid causes the amino acid functional groups to protonate, decreasing total solubility of the molecule and causing it to crash out of solution. Having reached the pH at each step which would cause precipitation, the only explanation for the

low results were the expected degradation of lipopeptides by bacterial growth during slow autotitration without temperature control at above room temperature.

To remove the protein contaminates, a two-step precipitation technique could be employed where the mass of unwanted proteins in the fraction was first precipitated at pH 4, with the loss of some lipopeptides, followed by Antifungal recovery at pH 2. Using this two-step approach towards lipopeptide purification and isolation, the recovery rate for antifungal lipopeptides was 3.09% ± 0.53 and 5.35% ± 1.85 for surfactin.

HPLC analysis also concluded precipitation trends remained constant between both the antifungals and surfactin (Figure 4-20) and thus little to no selectivity was present to separate antifungals from surfactin via this route.

Due to the lack of selectivity between antifungals and surfactin combined with low recovery of an already low yield product, other methods for isolation and purification were investigated.

4.6.2 Ammonium Sulphate Precipitation

Salt precipitation is used in a variety of biotechnological processes for the separation and purification of compounds from media. In this study salt precipitation was investigated for its ability to separate the complex composition of culture media into individual compounds and isolate purified lipopeptides.

Concentrated crude supernatant from *Bacillus amyloliquefaciens* was split into 5 mL aliquots and the salt concentration increased through addition of saturated $(NH_4)_2SO_4$ and the precipitate pellets isolated through centrifugation.

Looking at visual precipitation, the highest visual mass of precipitate was collected in fraction 2, at 15 ± 0.01% salt concentration, which had a large amount of golden-brown precipitate that formed. From fraction 5 onwards, >40% salt concentration, no more foaming occurred and it became very difficult to precipitate the pellet as the protein had the tendency to flocculate into flocs which only settled to the bottom if left to do so naturally. Centrifugation resulted in precipitate floating to the top and was removed using a glass pipetted followed by sequential centrifugation to remove all the liquid.

During preparation of the HPLC samples, the precipitated pellets resuspended in n-butanol displayed excessive foaming from fractions 1-4, approx. 10% - 40% $(NH_4)_2SO_4$, even with gentle vortexing. Foaming is indicative of the presence of lipopeptides and the amount of foaming found in each fraction, in decreasing order was 2 > 3 > 4> 1.

The discussion of HPLC results for lipopeptide and SO_4^{2-} concentrations follows and the results to be discussed are given in Figure 4-21.

No precipitation of lipopeptides was observed at 0% salt addition and recovery of antifungal lipopeptides increased slightly to 3.18 ± 0.48% at 6.00 ± 0.71% salt concentration in fraction 1. Further increasing the salt concentration from 6 ± 0.71% to 15 ± 0.01% resulted in the majority, 75.41 ± 1.65%, of antifungal lipopeptides to precipitate followed by another 17.23 ± 0.74% being recovered in fraction 3 at 24 ± 0.26% salt concentration. The amount of precipitation decreased significantly after fraction 3 with 3.75 ± 0.48% being recovered in fraction 4, 34 ± 0.88% salt concentration, and less than 0.1% recovery for fraction 5-8, >34% salt concentration.

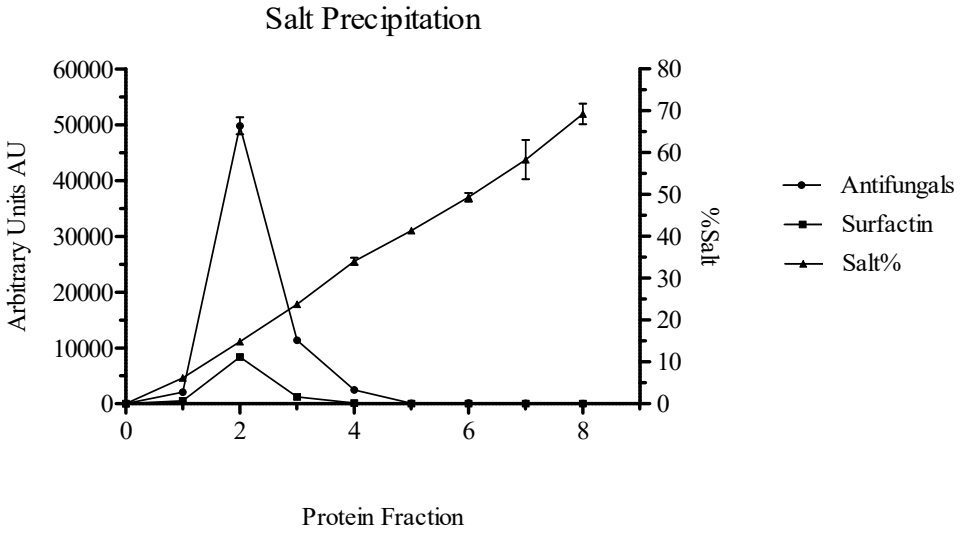

Figure 4-21 Lipopeptide isolation through ammonium sulphate salt precipitation (n=2). $(NH_4)_2SO_4$ concentration was increased linearly causing salt precipitation of surfactin and antifungals at fraction 2, 15 ± 0.01% saturated $(NH_4)_2SO_4$.

The same trend was observed for surfactin with the majority of surfactin, 81.96 ± 0.02% being recovered at 15 ± 0.01% salt concentration. Thus little to no selectivity between antifungal and surfactin lipopeptides where observed, but $(NH_4)_2SO_4$ did yield a high recovery rate of 99.05 ± 0.01% for lipopeptide isolation and presents an attractive route for bulk recovery of antifungal lipopeptides and purification from media components.

Incorporation of selectivity between antifungals and surfactin may be possible in combination with other downstream isolation methods to take advantage of the high yield from salt precipitation, but optimisation of the process parameters for salt precipitation was beyond the scope of this study and should be further investigated in future experiments. Also, co-precipitation of other media proteins could not be tested and thus purity of the isolated lipopeptides needs to be investigated in future studies

4.6.3 Solvent Extraction

Antifungal lipopeptide extraction through solvent extraction was investigated for its ability to separate and isolate antifungal lipopeptides from surfactin and other media components, this study being the first know study to report on these values.

Solvent extraction was carried out as a “single-step” solvent extraction procedure, using 7 different organic solvents (1-butanol, 2-butanol, isobutanol, dichloromethane, diethyl ether, tert-butyl-methyl-ether (TBME) and n-hexane) at 50:50 ratios of solvent to sample (1ml solvent to 1ml cell free supernatant). Solvents 1-butanol, 2-butanol, isobutanol were analysed in triplicate while 1-butanol, dichloromethane, diethyl ether, TBME and n-hexane experiments were tested in quadruplicate. The data from the results were used to elucidate on the migration of each family of lipopeptides between the two separate phases and calculate the distribution ratios and solvent selectivity factors to isolate and separate antifungals from the media constitutes.

The results for the migration of lipopeptides between the solvent and water phases are presented in Figure 4-22 for discussion.

Butanol samples achieved the highest recovery of antifungals in the solvent phase at 246 ± 20 AU and the remainder of 5 ± 1 AU in the water phase. The inverse was

found to be true for the other solvents where most of the antifungals, contaminated with other media compounds, remained in the water phase at 209 ± 4 AU, 167 ± 5 AU, 159 ± 2 AU and 102 ± 10 AU for n-hexane, diethyl ether, TBME and dichloromethane respectively.

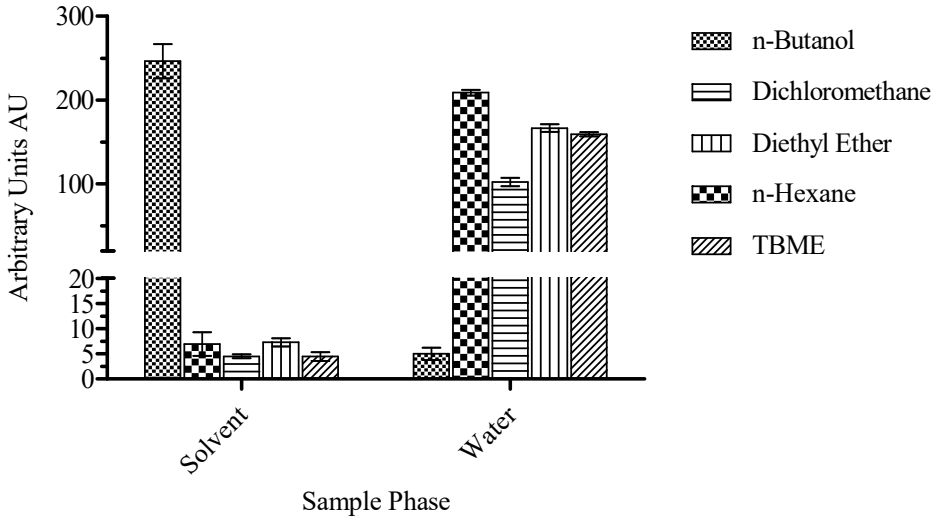

Figure 4-22 Antifungal lipopeptide isolation through organic solvent extraction (n=4).

Examining surfactin isolation (Figure 4-23), a similar trend was observed for butanol as with antifungal lipopeptides where most of the surfactin (>99%) moved into the solvent phase, 55 ± 4 AU, and only a small quantity was lost to the water phase, 0.43 ± 0.11.

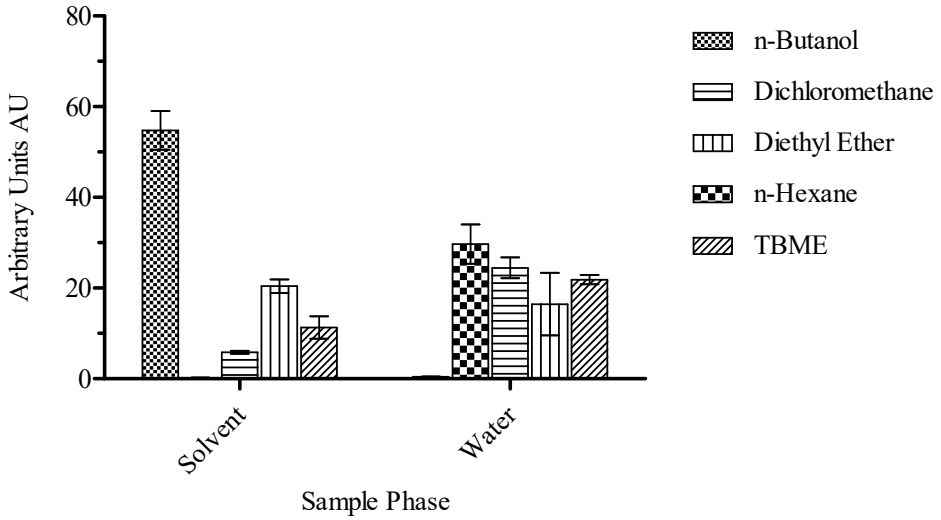

Figure 4-23 Surfactin lipopeptide removal through solvent extraction (n=4).

It is worth noting that extraction with the organic solvent n-hexane (Figure 4-23) resulted in virtually no migration of surfactin into the solvent phase, 0.18 ± 0.04 AU, compared to the other solvents at 20.41 ± 1.5 AU, 5.85 ± 0.70 and 11.30 ± 2.47 AU for diethyl ether, TBME and dichloromethane respectively. This finding, where 99.4 ± 0.2% surfactin remained in the water phase, will be further discussed in conjunction with the separation factors and lipopeptide recovery rates later in the chapter.

Apart from n-butanol, other butanol isomers where also investigated for their ability to extract antifungal lipopeptides and were found to result in similar trends (these results are displayed in Figure 4-24 and Figure 4-25).

Antifungal lipopeptides migration into the solvent phase remained constant for the three isomers 1-butanol, 2-butanol and isobutanol (Figure 4-24), but similarly to n-hexane the solvent isobutanol was found to also allow less surfactin to migrate into the solvent phase, 37 ± 0.42 AU (Figure 4-25), compared to 1-butanol and 2-butanol at 55 ± 4.3 AU and 64 ± 0.43 respectively. These findings, together with that of n-hexane, will be further later in the chapter.

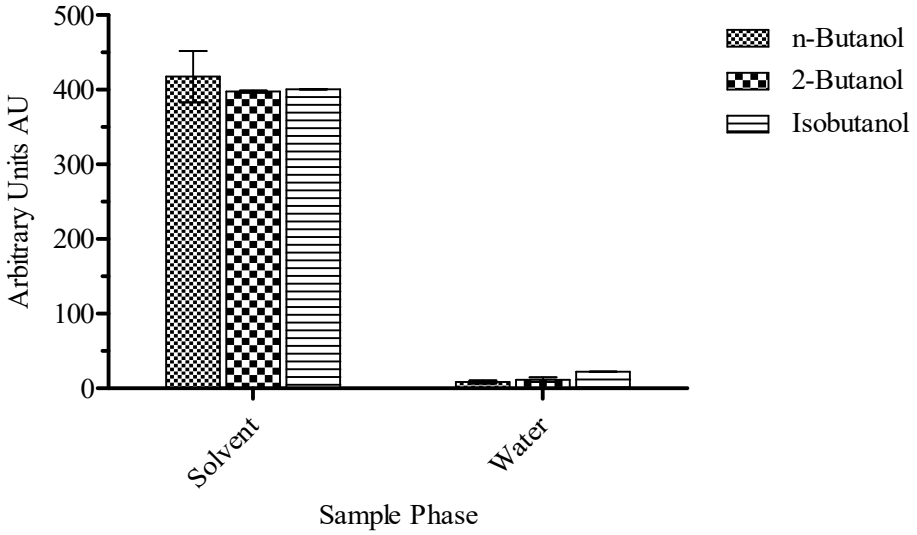

Figure 4-24 Antifungal lipopeptide isolation comparison between different butal isomers (n=3)

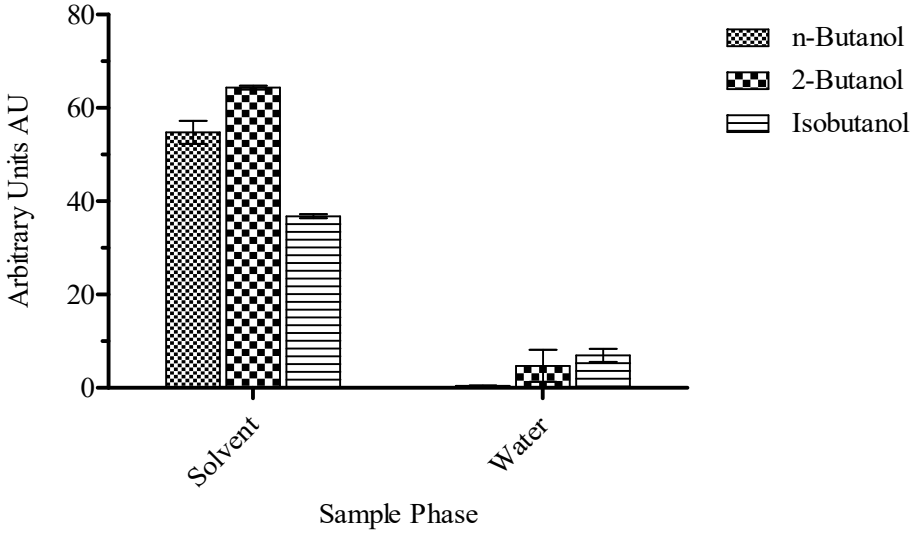

Figure 4-25 Surfactin lipopeptide removal comparison between different butanol isomers (n=3).

Using the results discussed above and Equation 4 in the methods section, the distribution ratios for antifungals and surfactin (Figure 4-26) as a ratio of lipopeptides in the organic phase vs the amount in the water phase were calculated. This allowed for comparison between lipopeptide distributions in the two-phase sample.

Figure 4-26 displays the distribution ratio of each solvent as a group with the ratio of antifungal lipopeptides presented left and surfactin right. Butanol isomers had ±100 fold greater migration of lipopeptides than diethyl ether and almost 1000 – 10 000 fold greater migration of lipopeptides than TBME, dichloromethane and n-Hexane. Thus butanol would seem to be able to isolate a large quantity of lipopeptides in a single extraction step and n-hexane the least amount. Solvents n-butanol, dichloromethane, diethyl ether and TBME displays visible selection towards surfactin migration into the organic solvent phase while 2-butanol, isobutanol and hexane displays selectivity towards antifungal lipopeptides and less towards surfactin.

To confirm the ability of each solvent as a viable solvent selection strategy to separate and isolate antifungal lipopeptides from surfactin and other media components, the distribution ratios of antifungal lipopeptides and surfactin was used in Equation 5 to calculate and quantify the selectivity for antifungal lipopeptides above surfactin during extraction. The results of these calculations are presented in Figure 4-27.

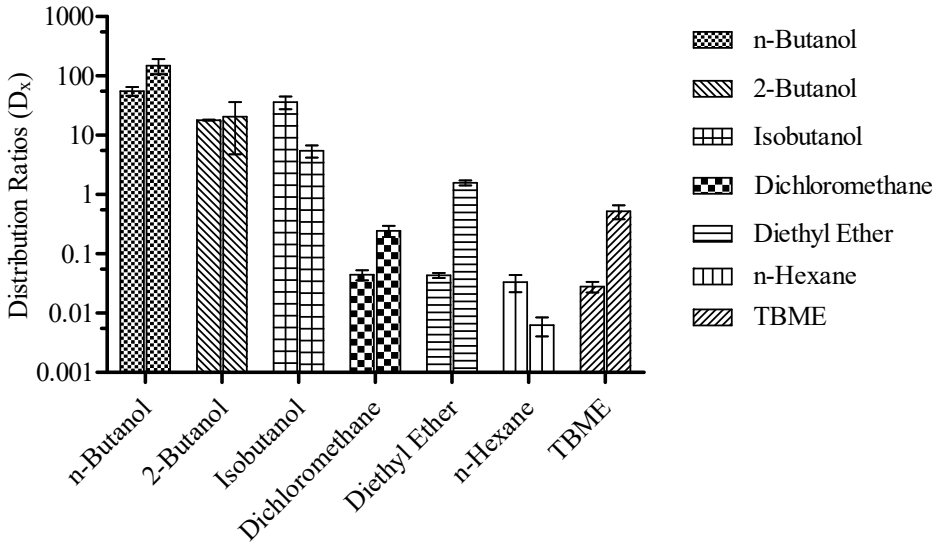

Figure 4-26 Comparison of the migration factor of different organic solvents and their individual distribution. The graph displays antifungals (left) and surfactins (right) for each solvent (n=4, 2-butanol and isobutanol n=3).

It was found that isobutanol and n-hexane had the highest separation selectivity towards antifungals, $\beta_{isobutanol}$ = 6.73 ± 1.69 and β_{hexane} = 5.31 ± 0.92, followed by significantly lower β_x values for 2 butanol, 1 butanol and dichloromethane at 1.32 ± 0.97, 0.40 ± 0.15 and 0.19 ± 0.07 respectively. This revealed isobutanol and n-hexane as attractive solvents for solvent extraction processes.

Looking at the overall recovery rates of the 7 organic solvents (Figure 4-28), butanol isomers had the highest recovery during a one-step isolation where >98% recovery was achieved. The other solvents, dichloromethane, diethyl ether, n-hexane and TBME all displayed low recovery during a one-step isolation, between 2-4%, and thus multiple isolation steps in series could improve this.

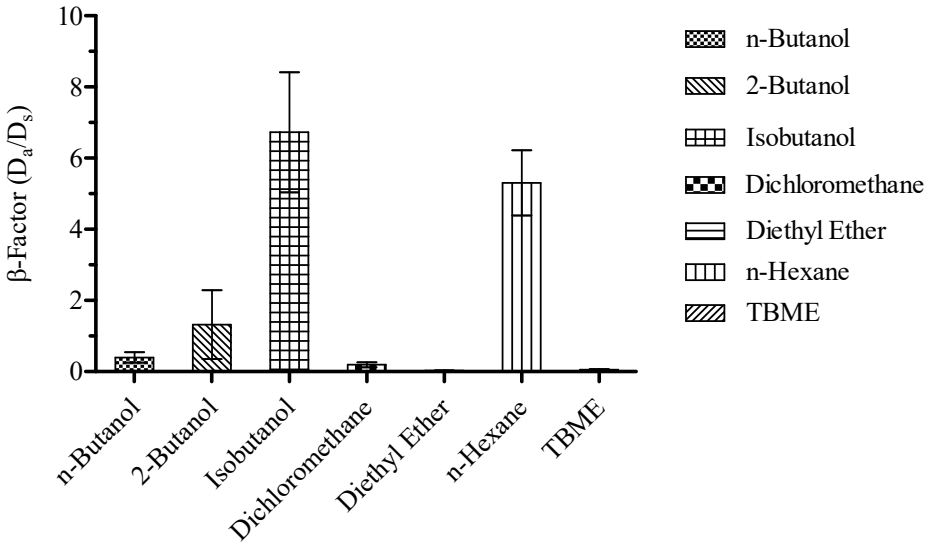

Figure 4-27 Solvent selectivity (β-factor), derived from equation 5, for comparison of each solvent to favour antifungal lipopeptide extraction above surfactin extraction by calculating $D_{antifungal}/D_{surfactin}$. (n=4, 2-butanol and isobutanol n=3)

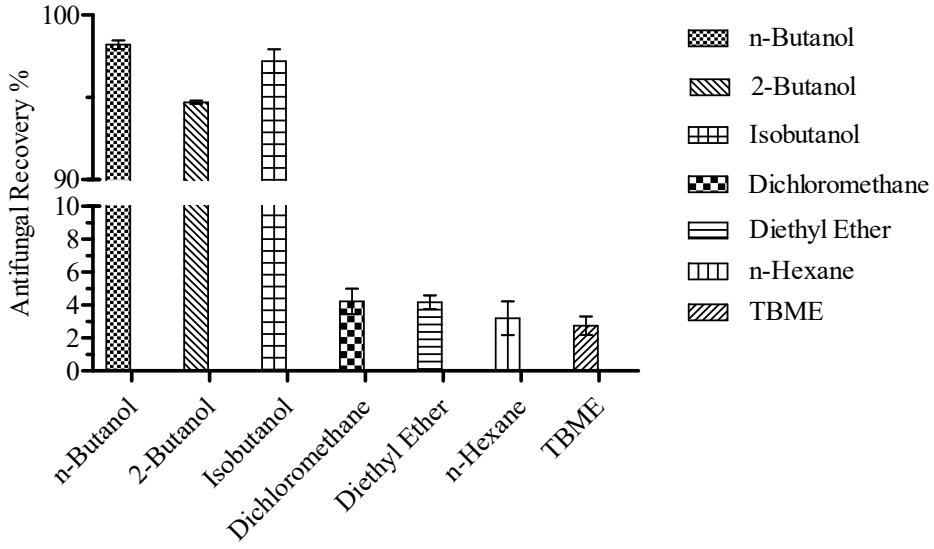

Figure 4-28 Comparison of percentage recovery of antifungal lipopeptides for each solvent (n=4, 2-butanol and isobutanol n=3)

Comparing the highest sample results of solvent extraction (n-Butanol) with salt precipitation (15% salt) and total acid precipitation, Figure 4-29, revealed solvent extraction using n-butanol presents the most favourable method for bulk lipopeptide isolation as a single step. The selectivity for any of these three methods, i.e. n-butanol as solvent, salt- and acid precipitation, still remains low as can be observed in Figure 4-29.

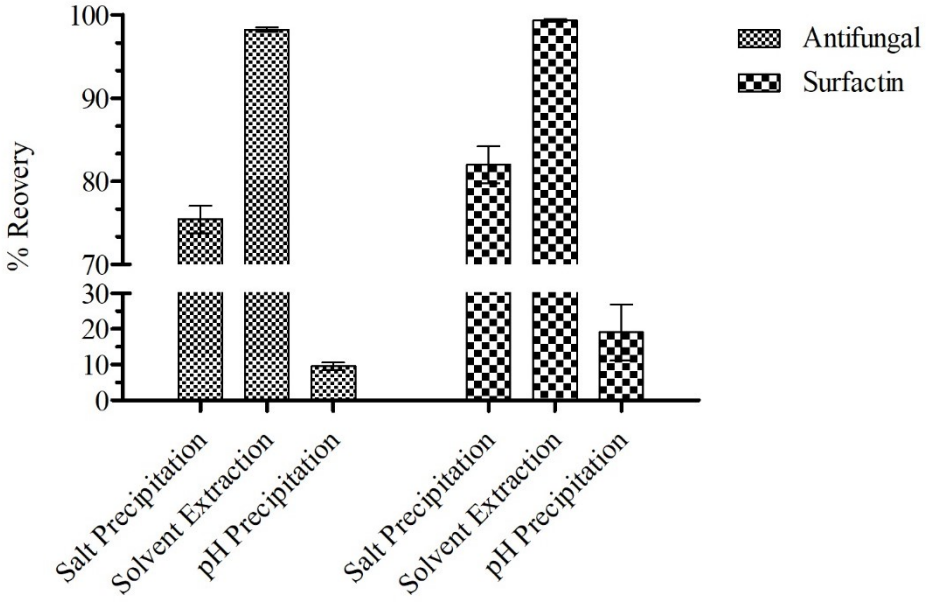

Figure 4-29 Comparison of lipopeptide yield from salt precipitation (15% saturated salt fraction), solvent extraction (n-Butanol) and acid precipitation (Total)

The results for all solvent used in solvent extraction revealed an opportunity to use organic solvents as a method for crude separation and isolation of antifungals from surfactin and other media proteins during extraction.

However, the migration (Figure 4-22) and recovery rate (Figure 4-28) for solvents such as n-hexane into the organic solvent is low and this meant that to benefit from the obtainable selectivity factor β_{hexane} (Figure 4-27), more than one separation step would be needed for complete extraction to be achieved which could increase the process cost, but with the benefit of high antifungal separation from surfactin in the final isolate.

Isobutanol on the other hand has been found to display comparable selectivity to n-hexane with the added advantage of >1000 fold increase in migration of antifungals (Figure 4-26) into the organic phase during a single isolation step and would prove to be the most reasonable solvent route towards high yield, antifungal lipopeptide isolation and subsequent purification strategies.

Due to time limitations, the optimisation of this was beyond the scope of this project and needs to be addressed in future studies

4.7 BIOLOGICALLY ACTIVE LIPOPEPTIDE ISOLATION AND IDENTIFICATION

4.7.1 TLC Bioautography Assay

One useful method particularly valuable in the screening and identification of unidentified compounds with antimicrobial activity is bioautographic assays. This method utilises a combination of TLC chromatography separation in conjunction with agar diffusion assays. Through this approach the R_f value, a unique "fingerprint" to each antimicrobial compound, could be determined where inhibition zones were observed. The compounds identified could then be isolated from the plates and further studied and characterised using other analytical techniques.

Partially purified and concentrated lipopeptides from n-Butanol solvent extraction were spotted and chromatographically separated on preparative TLC plates. Afterwards, plates were dried under vacuum at 40°C and covered with a slab of PDA agar inoculated with to 1% (v/v) solution of *Penicillium* spp. and *Botrytis* spp. spores.

The plates were cultivated at room temperature for 4 days and inspected for inhibition zones. A clear inhibition zone was observed between regions R_f 0.71 and R_f 0.87 (Figure 4-30, top photo) where neither the *Penicillium* spp. nor the *Botrytis* spp. spores were able to germinate and proliferate into mycelial colonies. Some growth was observed in the region R_f 0.94, where another compound had migrated to.

UV visualisation was employed to visualise all proteins, which include lipopeptides, present on the plate. The Compounds at UV_{380nm} on the TLC plate (Figure 4-30, middle photo) showed that three distinct regions, with R_f values of 0.71, 0.87 and 0.94, are proteins. The highest concentration, as a function of band brightness, was found at R_f 0.87 which was evidently also the centre point for inhibition of the bioatography assay.

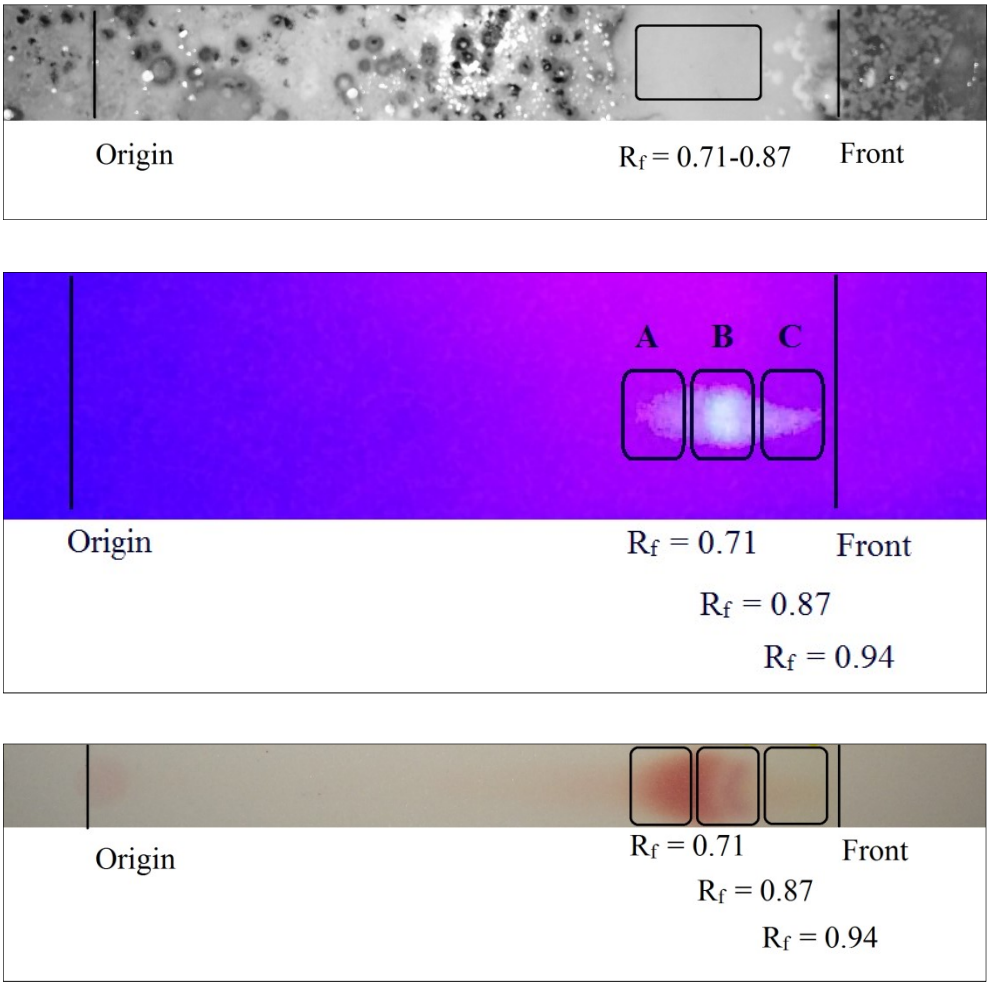

Figure 4-30 TLC crude purification and bioautography assays; top) Bioautiographt assay and R_f values for the inhibition zone observed, middel) UV visualization of a duplicate plate at 380nm and bottom) Ninhydrin 0.3% (v/v) development for amino acid detection.

Water misting of the plates to analyse for the fatty acid chains displayed hydrophobic properties of the compounds R_f 0.87 – 0.94 due repelling of the water indicative of possible lipopeptides being present in this region.

Ninhydrin was sprayed on the plates to test which of the proteins found present from UV results could be cyclic peptide peptides, which would not stain after development. The results are given in Figure 4-30, bottom photo, and shows a clear band in the middle of the spot at R_f 0.87 and no staining in R_f 0.94, thus

indicating the presence of compounds in protein of nature (UV findings) and having no free amino acids.

It could thus be concluded from UV, water and ninhydrin visualisation that both the unstained band at R_f 0.87, the centre of observed inhibition, and the compounds in the region of R_f 0.94 are likely hydrophobic proteins, as evidenced by the water and UV results, that contain no free amino acids, demonstrated by the ninhydrin results, and therefore the amino acids present must be organised in a closed structure, suggesting cyclic amino acid rings present and thus all these results together conclude cyclic lipopeptides are located in these regions.

Due to one inherent disadvantage of the diffusion assay approach, namely the inevitable diffusion of a compound from the centre point of administration to the outside in a agar media and taken the time frame of four days for this to occur, that it is likely that the compounds of R_f 0.87 might have migrated outwards and caused the spread of inhibition observed. This was also supported by the observation that at 7 days growth increased towards *Rf* 0.87 (data not shown), but no growth was achieved at R_f 0.87. It is still impossible to exclude the possibility that other compounds in the region of R_f 0.71 – 0.87 may contribute to the antifungal activity observed, but an assay with better resolution would be needed or the compound separated individually through HPLC, which was beyond the scope of this project.

It was thus decided for the scope of this research project and based on the concrete findings of the different visualisation techniques that compound(s) R_f 0.87, the centre point of inhibition, being a hydrophobic protein molecule with a possible amino acid ring structure, was the most likely candidate for isolation and further investigation.

The bands at the R_f 0.71, 0.87 and 0.94 were isolated from TLC plates and extracted with acetonitrile and analysed with HPLC to identify the lipopeptide(s) present. RP-HPLC data revealed the most abundant type antifungal lipopeptide present in isolate R_f 0.87 belonged to the Iturin A family at 497.58 mAU*min (85.63% of total sample) and to a lesser extend a few other unidentified lipopeptides of 83.51 mAU*min (14.37 % of total sample)(Figure 4-31). This finding is supported by the findings of correlation studies (Figure 4-19) and conclusive with research by Arrebola, et al. (2010) that also identified iturin as the lipopeptide involved in *Aspergillus* and *Alternaria* inhibition.

The composition of R_f 0.71 comprised mainly of other proteins unable to be identified via the RP-HPLC method with no measurable quantity of lipopeptides present, which supports the ninhydrin results for the lack of cyclic lipopeptide structures, while R_f 0.94 contained mainly surfactins and was also supported by the ninhydrin and water misting results.

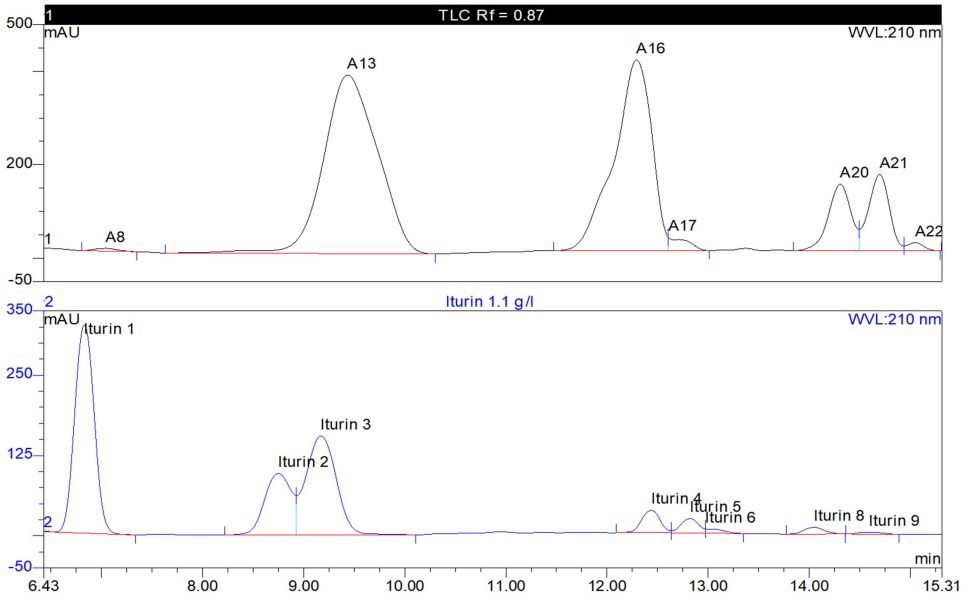

Figure 4-31 HPLC chromatographs for TLC isolation R_f 0.87 (top) in comparison to Iturin A standards (bottom)

Data available suggests surfactin to have low inhibition towards fungi (Alvarez, et al., 2012) with the antifungal activity from surfactin contributed to synergism from the presence of Fengycin and/or Iturin. Therefore, R_f 0.87, identified as Iturins, must be the main compounds responsible for the inhibition observed. The diffusion of antifungals into the region from R_f 0.87 to R_f 0.94 thus had a minor contribution from synergism, with surfactin and hence the small amount of growth visible in border between R_f 0.87 to R_f 0.94 regions and activity decreasing as surfactin concentration increase towards >R_f 0.94 (Figure 4-30, top photo).

4.7.2 MIC Determination of Efficacy

Now that the compound of interest was confirmed to be Iturin A, the MIC value for this lipopeptide family was investigated. Such MIC values will be needed to further optimise process design with regards to lipopeptide concentration for the final product and help future researchers in developing similar efficacy studies.

Lipopeptide concentrate from solvent extraction, containing 2.0 g/L Iturin, was used to perform dilutions in serie in a 96 well microtire plate. This was done by adding 300 µL of the concentrate in a well followed by 24 subsequent dilutions to fall in the range of 1.0 g/L – 0.04 g/L with a 1:1 dilution by broth addition. A positive control, water, with no antifungals was added for quality control.

Malt extract broth was prepared, malt extract sucrose, and half inoculated with spores from *Penicillium* and the other half *Botrytis*. Broth was added to the diluted Iturin in each plate, 1:1, and the plates incubated at room temperature for 4 days.

No definitive MIC could be derived as no growth was observed in all dilutions compared to the positive control which did grow. Thus inhibition is still achieved at 40 mg/L Iturin and the range should be extended further obtain MIC.

Chapter 5

GENERAL DISCUSSION

The work presented in this study aimed to provide evidence that *Bacillus* spp. lipopeptides are effective biocontrol agents that have the potential to replace chemically synthesised pesticides that are currently in use. These biocontrol agents were evaluated on their ability to act against phytopathogens responsible for postharvest disease found in, but not limited to, the South African table grapes industry.

Furthermore, the study aimed to also inform on the specific lipopeptides responsible for any antimicrobial activity against the phytopathogens and on possible methods for lipopeptide isolation and purification for downstream processing.

To build a phytopathogen culture bank for experiments, 79 crude isolates of fungi were made and through using 5 consecutive sub-culturing rounds on three types of agar plates, PDA, MEA and NA, 59 pure cultures were achieved. Of these, 16 out of 18 that were sequenced successfully yielded DNA sequences for genus identification through nucleotide alignments. Using microscopy, plate morphology and individual DNA alignments, with additional inter-genus comparisons, identification of the phytopathogen genera were successfully achieved.

Of the sequenced fungi, 50% belonged to *Penicillium* spp., 33% to *Botrytis* spp. and 17% as *Aspergillus* spp., *Alternaria* spp. and *Lewia* spp. grouped. These isolates are responsible for noble rot, blue-green mould, bunch rot, black mould and leaf blight in the grape industry respectively. Thus *Bacillus* spp. lipopeptides could be tested against the two most common phytopathogenic phyla affecting the grape (*Botrytis* spp.) and citrus (*Penicillium* spp.) industries. A pure fungal culture bank of all 59 cultures was kept throughout the duration of the study. As a result of all the above, aim 1 set out in this study was met.

For the production of lipopeptides, four *Bacillus* strains were selected for screening experiments based on the spectrum of lipopeptide production recorded in literature. All cultures were successfully maintained in a culture bank throughout the study and thus fulfilling aim 2.

To culture the chosen *Bacillus* strains, it was set out to develop a theoretical "one-size-fits-all" media formulation from literature on different media elements. Following 9 different media formulations, the selected media was tested and it was found that most of the lipopeptides partitioned to a precipitate. The media was adjusted to allow all the lipopeptides to be collected as fractions in the supernatant to simplify isolation, HPLC characterisation of lipopeptide production and efficacy studies.

This study was the first known research approach to characterise the media lipopeptide concentrations as media fractions where the lipopeptides could be isolated, i.e. foam, supernatant, precipitate or cells fractions and to modify the media as such to isolate lipopeptides in a certain fraction. Calculating the lipopeptides present it was found that 85.92% of antifungals were found in the precipitate, 6.82% in foam, (4.65%) in supernatant and 2.62% in cells. Surfactin was found to contain 78.30% in the precipitate, 8.18% in foam, 10.51% in cells and 3.01% supernatant.

The results gave a new perspective on the dangers of reporting and drawing conclusions on single fraction concentrations, like supernatant, alone. This approach could also identify the cell fraction as a potential source of lipopeptides in downstream isolation strategies.

For radial diffusion assays crude lipopeptide mixtures of all the four *Bacillus* strains were obtained from culture sampling at different times in the growth cycle and were tested for their ability to inhibit the fungal phytopathogens isolated from

South African Grapes. Strain *B. amyloliquefaciens* was found to be highly effective against all the phytopathogens tested. This was also true for *Aspergillus* spp., species that are known producers of carcinogenic toxins on contaminated food, particularly in poorer countries. The activity observed towards the tested fungi was mostly fungicidal (Figure 4-13) since clear inhibition halos were recorded under the microscope with the exception of activity towards *Penicillium digitatum* (Figure 4-15) being characterised as static inhibition. This was due to growth in the halo from spores that were being inhibited after germination and normal growth resuming when culture plug of inhibited spores from *Penicillium digitatum* was moved to lipopeptide free media. This result could also mean that the doses tested were too low to achieve fungicidal activity in the case of *Penicillium digitatum* and higher doses might overcome the cellular defence mechanism.

The lack of inhibition activity for the other three *Bacillus* strains was attributed to the optimised media only supporting *B. amyloliquefaciens* to grow well and produce significant antifungal lipopeptides to study. Consequently, *B. amyloliquefaciens* was chosen as the candidate organism for further study in lipopeptide production and isolation based on the fact that it could produce antifungals on the developed media, but this is not to say that the other 3 strains could not have equally performed should the correct media composition have been found for each strain. However, an investigation of optimal media for each individual strain in isolation was not the focus of this work, and so the use of *B. amyloliquefaciens* as a study organism was sufficient for this study to reach each of the goals set out in the beginning.

A mathematical approach was employed to identify *B. amyloliquefaciens* lipopeptides responsible for antifungal activity through correlating antifungal activity with each lipopeptide homolog's concentrations measured in *B. amyloliquefaciens* during shake flask culturing. The results concluded the activity was most probably due to antifungals in the Iturin family. To confirm these results experimentally, Butanol extracts were concentrated on TLC plates and TLC separation coupled to bioautographic assays confirmed the iturins were responsible for activity and displayed overlap with iturins 3, 4, 5, 8 and 9 from an Iturin HPLC standard purchased.

Crude lipopeptide mixtures from active culture media were subjected to acid, salt and solvent extraction methods in the hopes of separating the lipopeptide families

from each other. With the obtained results the study aimed to lay down a foundation and characterise the extraction efficiency and determining the ratios of each lipopeptide homolog separating into each phase for different solvents reported useful in solvent extraction. The results reported on isolation and separation efficiency was the first known research approach that differed from conventional research which only focused on total lipopeptide isolation.

Salt and solvent extraction showed the most promise as bulk isolation methods, but solvent extraction using isobutanol or n-hexane could be applied to isolate antifungals from surfactin.

The study was ended with all being met.

By fulfilling all four aims set out in the beginning this study it was established that *Bacillus* lipopeptides are promising candidates for further research and development towards a biologically benign and green alternative to chemical pesticides in many crops applications, including the South African Grapes and Citrus industries.

5.1 Final Comments and Future Research Recommendations

- Further research into media formulations for the other 3 strains, *B. licheniformis* DSM 13, *B. subtilis* ATCC 21332 and *B. subtilis spizizini* DSM 347, could yield positive results and should be included in future research towards this goal.

- Radial Diffusion assays were used to allow large scale screening for antifungal activity. More sensitive assays that are not influenced by diffusion gradients through the agar and subsequent dilution of antifungal compounds should yield better results for sensitivity studies.

- Although the bioautographic assay was a powerful tool in the determination of the lipopeptides families responsible for antifungal activity, the lack of poor resolution to separate and isolate individual iturin homologs, chromatically and manually from plate, could contribute to the results of multiple

iturins being assigned to the observed inhibition activity. Future studies should look into using biological assays specifically using individual iturin homologs and also testing individual homolog MIC.

Chapter 6

CONCLUSION

The study presented focused on investigating the prospect of antifungal lipopeptides from four *Bacillus* spp. as potential candidates for use in future development of a cell-free biocontrol agent to be effective against phytopathogens responsible for postharvest disease. The screening criteria were based on the ability of different *Bacillus* species to produce antifungal lipopeptides which could inhibit phytopathogens isolated from post-harvest grapes.

Furthermore, the study aimed to inform on homologs produced during lipopeptide production, the specific lipopeptide homologs responsible for the observed antimicrobial activity against phytopathogens and on possible methods for lipopeptide isolation and purification.

The key findings of the study are documented as follows:

- **Most of the phytopathogens isolated from SATGI grapes were filamentous fungi.**

The grouping of isolated filamentous fungi by genera could be divided into *Botrytis* 33%, *Penicillium* 50% and *Alternaria*, *Aspergillus* and *Lewia* as 17% combined. These phytopathogens are responsible for noble rot, blue-green mould, bunch rot, black mould and leaf blight in grapes respectively. The main phytopathogens contributing to losses and the grape (*Botrytis* spp.) and citrus (*Penicillium* spp.)

industry could subsequently be evaluated. Other crops affected through the same phytopathogens, as with *Aspergillus* on maise or *Penicillium* in citrus fruit could benefit from lipopeptides as biocontrol agents.

- **ITS primers were effective in determining the genus of fungi.**

Through the use of ITS 4 and ITS 5 primers the genera of 16 out of 18 fungal cultures could be determined in combination with morphology studies. More detail identification, i.e. specie level, would have been beneficial in total identification of the specific specimen and would require specie specific primers to be designed and used in future studies.

- **Not all the *Bacillus* strains could be cultivated on a single defined media formulation.**

Of the four strains tested, only *B. amyloliquefaciens* DSM 23117 could thrive and produce and array of 47 lipopeptides, 169 mAU*min antifungal lipopeptides, on the media formulation developed. Thus better formulations are needed to address the unique nutritional needs of the other strains in order to be effectively evaluated as potential candidates.

- **Yeast extract was an important requirement during shake flask culturing.**

Media formulations with yeast extract and additional inorganic nitrate resulted in better growth and higher lipopeptide production in formulations containing 0.5g/L yeast extract and 4g/L NH_4NO_3 which yielded 2.534g/L surfactin in media formulation experiments. The results could be explained due to the buffering effect yeast extract has on pH control and be a source of amino acids, nitrogen, minerals and vitamins.

- **Lipopeptides could be kept in the supernatant for isolation with media formulations incorporating a buffer to work against the pH drop.**

Most of the lipopeptides precipitated out due to severe pH drop in the base media. Using an appropriate phosphate buffer with sufficient buffering capacity the antifungal lipopeptides were successfully kept in the supernatant for isolation and further experimentation. Low pH causes functional groups on the hydrophilic

moiety to become hydrogenated; decreasing solubility until self-aggregation and precipitation takes place.

- **Cells fractions were found to be a source of lipopeptides for product isolation and optimisation strategies.**

Cells could also be a source of lipopeptides, especially for studies focussed towards surfactin isolation and needs to be taken into account during optimisation strategies for large scale production.

- ***Bacillus amyloliquefaciens* DSM 23117 was proven an effective biocontrol agent towards phytopathogens isolated from South African table grapes, even in the crudest form.**

Results from four *Bacillus* strains cultured revealed *B. amyloliquefaciens* as a superior producer strain of antifungal lipopeptides and could inhibit the growth of all 9 fungal phytopathogen strains, *Botrytis*, *Penicillium*, *Alternaria*, *Aspergillus* and *Lewia*, subjected to crude and purified supernatant extracts.

Secondly, these results revealed the potential of using crude cell-free isolates instead of a highly purified product which results in a lower production costs and simplifies the process of downstream isolation. These results point towards this specific strain to be included in further downstream process optimisation studies.

- **Lipopeptides exibited fungicidal activity towards filamentous fungi, with the exception of *P. digitatum* which resulted in static inhibition as long as lipopeptides were present.**

From closer investigation of radial diffusion inhibition halos under the microscope it was determined that *bacillus* lipopeptides could destroy mycelia and spores of filamentous fungi and inhibit further proliferation of the fungus. However, *P. digitatum* was only statically inhibited during spore germination and spore growth could resume if lipopeptides were removed. The results are explained from different ratios in membrane components, such as cholesterol, that buffer these antifungal actions through stabilizing the membrane. Combing research on spore and cell membrane composition of the isolates would explain how *P. digitatum* resists cell damage at the concentrations tested. Thus, the observed effect could also be dose dependent and call for higher doses to be investigated on *P. digitatum*.

- **The Iturin family is responsible for antifungal activity and is a crucial target for incorporation into isolation strategies towards antifungal biocontrol agents.**

Iturins are documented to cause pore formation and together with membrane structure analysis, future studies focussed towards membrane interaction models could further explain the mechanisms involved. This study lacked testing of synergism and surfactin could have played a contributing role in activity.

- **MIC is below 40 mg/L total Iturin**

Using MIC assays it was determined that phytopathogens are effectively inhibited at 40mg/L. No definitive MIC could be determined, thus the experiment needs to be repeated at lower concentrations to determine more specific MIC values. MIC values would guide downstream processes toward product optimisation in that MIC would determine a working concentration for maximum efficacy and secondly save cost through not having unnecessarily concentrated product.

- **Salt Extraction yields high isolation, but offers limited separation capability**

The result of salt precipitation from ammonium sulphate offered a means of isolating lipopeptides in bulk with >99% and could be used as a first step for lipopeptide isolation from culture media. However, it offered little separation capabilities to separate surfactin from antifungals with 75.41 ± 1.65% of antifungal lipopeptides and 81.96 ± 0.02% surfactin recovered collectively at 15 ± 0.01% salt saturation. Thus salt precipitation could be employed as a first bulk isolation step followed by a second isolation step with high antifungal selectivity, like isobutanol and hexane extraction.

- **Solvent extraction through isobutanol yields low isolation, but offers high separation efficiency**

Using Isobutanol in solvent extraction yielded high separation efficiency for isolating antifungals from surfactin from culture media with $\beta_{isobutanol} = 6.73 \pm 1.69$. Thus, Isobutanol is an attractive solvent further investigation into downstream processes.

Through the use of various analytical and experimental methods and new approaches towards investigating downstream processing strategies, all the aims set out in this study were met. The findings of this thesis validated *Bacillus amyloliquefaciens* as an ideal candidate for antifungal lipopeptide production and identified Iturin as compounds of interest for incorporation into future studies. These include research to be conducted to understand parameters influencing antifungal lipopeptide synthesis and how to optimise large scale isolation of the lipopeptides using the methods applied in this study. Ultimately, the goal with such research in mind is to provide cost effective solutions towards mass production of a cell-free lipopeptide products that are just as effective and affordable as chemical pesticide.

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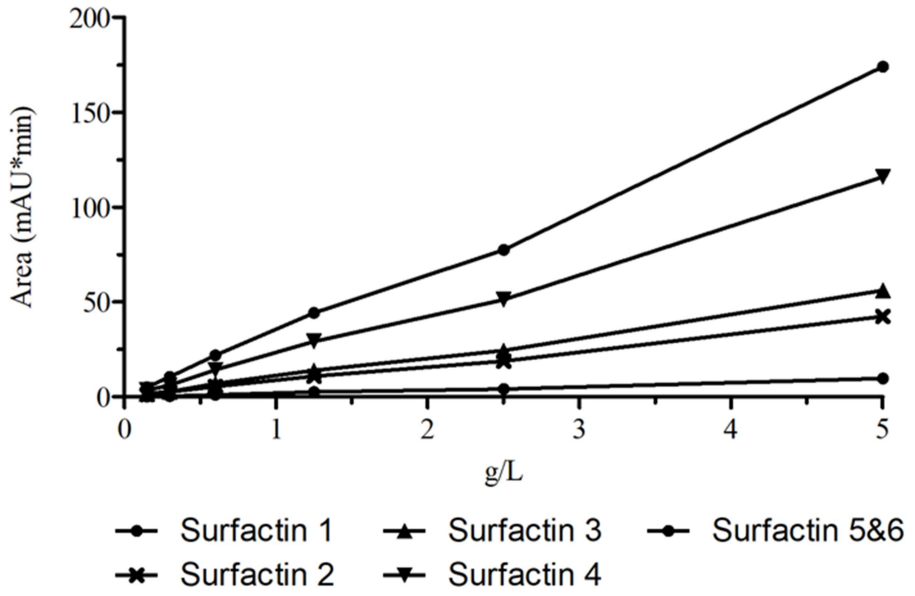
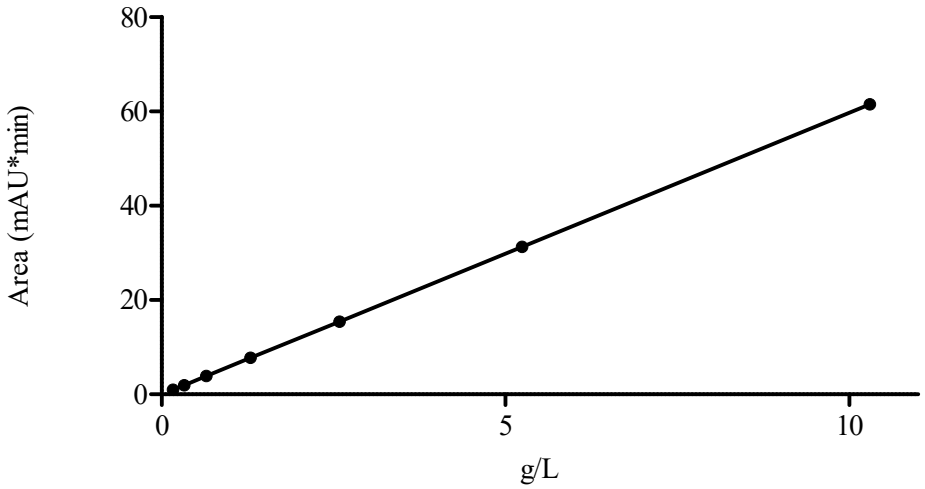
ADDENDUM A



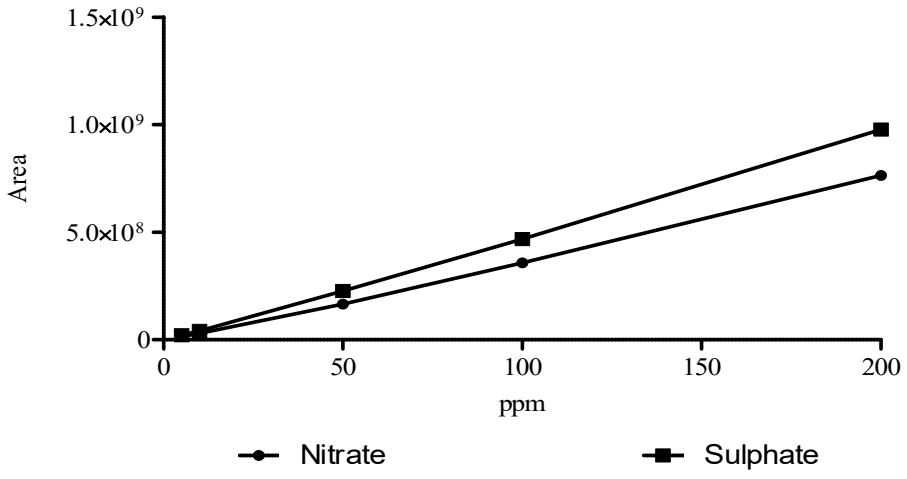
	Surfactin 1	Surfactin 2	Surfactin 3	Surfactin 4	Surfactin 5&6
Slope	*1.908 ± 0.08468*	*8.320 ± 0.2630*	*11.05 ± 0.3943*	*22.86 ± 0.7336*	*34.24 ± 1.055*
R square	*0.9922*	*0.9960*	*0.9949*	*0.9959*	*0.9962*

Figure A 1 Surfactin calibration curve used in HPLC analysis for determination of surfactin concentration in shake flask experiments.



	Glucose
Slope	*5.971 ± 0.0001720*
R square	*1.000*

Figure A 2 Glucose calibration curve used in HPLC analysis for determination of surfactin concentration in shake flask experiments.



	Nitrate	Sulphate
Slope	*3.849e+006 ± 83383*	*4.906e+006 ± 61260*
R square	*0.9986*	*0.9995*

Figure A 3 Nitrate and sulphate calibration curve used in HPLC analysis used for nitrate and ammonium sulphate concentration.

ADDENDUM B

I. Ammonium sulphate volume addition reference table

Desired Final Ammonium Sulfate Percent Saturation

Initial Ammonium Sulfate Percent Saturation	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95
0	0.111	0.176	0.250	0.333	0.429	0.538	0.667	0.818	1.000	1.222	1.500	1.857	2.333	3.000	4.000	5.667	9.000	19.000
10		0.059	0.125	0.200	0.286	0.385	0.500	0.636	0.800	1.000	1.250	1.571	2.000	2.600	3.500	5.000	8.000	17.000
15			0.063	0.133	0.214	0.308	0.417	0.545	0.700	0.889	1.125	1.429	1.833	2.400	3.250	4.667	7.500	16.000
20				0.067	0.143	0.231	0.333	0.455	0.600	0.778	1.000	1.286	1.667	2.200	3.000	4.333	7.000	15.000
25					0.071	0.154	0.250	0.364	0.500	0.667	0.875	1.143	1.500	2.000	2.750	4.000	6.500	14.000
30						0.077	0.167	0.273	0.400	0.556	0.750	1.000	1.333	1.800	2.500	3.667	6.000	13.000
35							0.083	0.182	0.300	0.444	0.625	0.857	1.167	1.600	2.250	3.333	5.500	12.000
40								0.091	0.200	0.333	0.500	0.714	1.000	1.400	2.000	3.000	5.000	11.000
45									0.100	0.222	0.375	0.571	0.833	1.200	1.750	2.667	4.500	10.000
50										0.111	0.250	0.429	0.667	1.000	1.500	2.333	4.000	9.000
55											0.125	0.286	0.500	0.800	1.250	2.000	3.500	8.000
60												0.143	0.333	0.600	1.000	1.667	3.000	7.000
65													0.167	0.400	0.750	1.333	2.500	6.000
70														0.200	0.500	1.000	2.000	5.000
75															0.250	0.667	1.500	4.000
80																0.333	1.000	3.000
85																	0.500	2.000
90																		1.000
95																		

Figure B 1 Table for calculating the total volume of ammonium sulphate addition to increase sample percentage salt saturation. (Sigma Aldrich Technical Bullitin A 4579, https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/a5479bul.pdf)

ADDENDUM C

Table C 1 Halo diameter (in mm) for efficacy studies using the selected cultures isolated from SA Crops and *B. amyloliquefaciens* lipopeptide samples in triplicate. Experiments were performed using *Bacillus* spp. lipopeptides sampled over the duration of 7 days by means of shake flask culturing.

	Lewia spp. (1)			*Botrytis* spp. (2)			*Botrytis* spp. (3)			*Penicillium* spp.(4)			*Alternaria* spp (5).			*Aspergillus* spp.(6)			*Botrytis* spp. (7)			*Botrytis* spp.(8)		
Triplicate	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Day 1	18	16	18	18	17	15	10	11	10	18	17	18	20	20	18	10	12	12	17	15	18	20	20	20
Day 2	18	20	18	12	13	13	12	12	13	12	14	12	20	20	20	14	12	10	11	12	14	16	17	16
Day 3	20	24	16	16	15	16	20	18	20	20	18	20	20	20	20	17	18	18	17	18	17	24	22	23
Day 4	22	20	22	18	18	18	18	20	20	14	12	12	16	14	14	14	16	10	20	15	20	20	14	20
Day 5	20	14	14	18	13	13	12	11	12	15	15	14	14	14	10	14	14	14	0	0	0	15	17	14
Day 6	20	20	20	9	8	8	0	0	0	18	17	17	20	20	16	10	11	11	0	0	0	12	12	12
Day 7	20	20	20	0	0	0	12	12	11	13	13	12	20	16	12	22	23	22	0	0	0	12	12	13